

DISSOCIATION OF B. ANTHRACIS

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INTRODUCTION

In recent years increasing attention has been given to studies dealing with variations in properties within a bacterial species and more particularly such variation that affect colony form. Following the publications of Arkwright¹ and DeKruif² a generalized type of work has followed in which the R and S forms of colonies have been obtained from pure cultures of various species of bacteria. Hadley³ has published an excellent review of the literature dealing with the various phases of this subject. The correlation of such properties as virulence, motility, morphological and immunological properties, etc., with colony form in many of the species studied, has aroused more than a passing interest in this field of bacteriological investigation.

The significance that has been attached to variation in properties of bacteria has been widely different. Certain workers including Pasteur⁴ and Preisz⁵ have recorded variation in virulence and morphological properties in cultures subjected to unfavorable conditions. They have spoken of such changed cultures as weakened or attenuated. Neisser⁶ has referred to variations in *B. coli mutabile* studied with Massini⁷ as mutations in the sense of DeVries. DeKruif² also interpreted his findings as mutations but in a sense defined by Dobell.* Hadley³ has attached considerable importance to the work of Enderlein, Löhnis, and Mellon and believes that there exists a life cycle for bacteria in which the various colony forms represent stages. No consensus of opinion exists as to the exact significance to be attached to such phenomena as have been described under such titles as variation, mutation and more recently as dissociation.

Throughout the extensive literature involving studies on the anthrax bacillus, numerous reports are to be found dealing with variations in this or that property

1. J. Path. & Bact., 1921, 24, p. 36.

2. J. Exper. Med., 1921, 33, p. 773.

3. J. Infect. Dis., 1927, 40, p. 1.

4. Compt. rend. Acad. d. Sc., 1881, 92, pp. 428-429.

5. Centralbl. f. Bakteriol., 1, O., 1911, 58, p. 510.

6. Ibid., 1, ref., 1906, 38, p. 98.

7. Arch. f. Hyg., 1907, 61, p. 250.

* "A permanent change however small it may be—which takes place in a bacterium and is then transmitted to subsequent generations."

of this organism. Early in bacteriological history Pasteur,⁴ Chamberland and Roux,⁸ and Gamaléia⁹ noted morphologic differences between the organisms of vaccine and virulent cultures.

Preisz¹⁰ in 1904 studied sporogenic and asporogenic races which could be differentiated by slight differences in color and structure of the colonies. This observation in difference of colony structure was the forerunner of more extensive work by Preisz,⁵ as well as others, which becomes of special importance in view of the present emphasis placed upon colony form in the recent developments on the subject of bacterial dissociation.

In 1908 Preisz¹¹ reported on further differences observed in the structure of anthrax colonies. He extended his observations in his laudable publication of 1911.⁶ In this paper Preisz showed that it was possible, starting with a "normal virulent strain of the anthrax bacillus, to obtain a series of variations by growing the culture in broth (without peptone) at a temperature of 42.5 C. These variants, so obtained, correspond closely to the variants previously reported by Preisz," as occurring in Pasteur vaccine cultures. The usual culture type described by Preisz as well as all earlier workers can, from its cultural characteristics, be designated as the R form. Preisz described one of his variants as giving rise to a homogenous, round colony. This, probably from its colony properties, could be designated as the S form. The latter form had a diminished virulence according to Preisz, while the former was fully virulent. Of no less significance, I believe, were the mucoid forms described by Preisz. One of these was a structureless, slimy growth characterized by adjacent colonies flowing together. The second mucoid colony had more or less of the usual rough anthrax structure with the addition of being mucoid. Colony forms were also described which no doubt were intermediate with respect to the roughness and smoothness, or the mucoid nature of the colony. The mucoid cultures killed mice from which normal anthrax cultures were recovered as well as the slimy forms inoculated. It is to be noted that Preisz also records *in vitro* dissociation of the smooth mucoid form into what would now probably be termed S and R cultures.

Markoff¹² the following year made a study of 53 strains of *B. anthracis*. From these he obtained, by plating methods, variants with respect to cultural and morphological properties. These variants were in general, not dissimilar to those described by Preisz. Markoff emphasizes the age of the culture, nature of the medium, temperature and moisture conditions and lastly the individual "Veranlagung" of the strain, as factors resulting in variations.

Baerthlein¹³ in a brief note stressing the fact of mutation among bacteria described two atypical forms of anthrax colonies. One was a transparent type tending to flow and coalesce with adjacent colonies (Preisz's structureless mucoid). The other may have been similar to Preisz's homogenous type and was described by Baerthlein as being small, sappy and of a cloudy yellow or white color. Both forms killed mice.

In 1912 Eisenberg¹⁴ also published his first paper dealing with sporogenic and asporogenic races of the anthrax bacillus. He was able to isolate such races from laboratory cultures and was also able to convert the one into the other. He observed some slight variation in colony structure depending on whether the strain was sporogenic or asporogenic. Eisenberg continued these studies and

8. Compt. rend. Acad. d. Sc., 1883, p. 96, p. 1088.

9. Ann. de l'Inst. Pasteur, 1888, 2, p. 517.

10. Centralbl. f. Bakteriöl., 1, O., 1904, 35, p. 280.

11. Ibid., 1908, 47, p. 585.

12. Ztschr. f. Infekt. d. Haustiere, 1912, 12, p. 137.

13. Centralbl. f. Bakteriöl., 1, ref., 1912, 54, Beilage, p. 178.

14. Ibid., 1, O., 1912, 63, p. 305.

reported his results in 1914,^{14a} giving the interpretation that these variations might represent in part modifications, and in part mutations. Mme. Henri¹⁵ in the same year reported having produced 5 morphological forms ranging from rods to coccoid shapes by exposing the anthrax bacillus to ultraviolet light. When these atypical forms were inoculated into guinea-pigs they reverted to the normal forms.

Bail and Flaumenhaft¹⁶ isolated from a guinea-pig previously inoculated with a partially attenuated strain of *B. anthracis* (grown at 42 C.), two forms giving distinct colonies. The one which they designated as β corresponds to the usual anthrax colony (R), while the description of the other form, designated by them as α agrees closely with the homogeneous colony of Preisc⁵ which is analogous to the so-called S colony forms in other bacterial species.

In all of the work mentioned so far the variations have been observed in laboratory cultures, vaccine cultures and cultures placed under attenuating conditions.

Hence Wagner's¹⁷ report carries with it the special significance that the atypical strain of *B. anthracis* was isolated from a fatal human case. This atypical form manifested itself by giving rise to small, compact colonies which Wagner designated as the B form in contrast to the normal or A form isolated at the same time. The excellent pictures accompanying this report enable one to identify this atypical type as belonging in general to the category of S forms, with respect to the current terminology of microbic dissociation. Wagner reports no difference in the pathogenicity of these two forms for the guinea-pig. On the other hand the S form (Wagner's B) killed mice in 13 hours while the R form isolated from the same patient required 72 hours and 80 hours, respectively, to kill two mice. It is to be noted that this is the first report in which greater virulence is attributed to the S form of the anthrax bacillus than to its R form.

Lutz¹⁸ working with a slightly virulent* strain of *B. anthracis* succeeded in obtaining under various aging conditions, coccoid forms of the anthrax bacillus. These forms in time revert to the normal form in all of their "physiological and biological properties." He denies that these coccoid forms are degeneration forms on the basis that they possess an intense life energy.

In 1924 two reports were made concerning the findings of Wagner's B type. Gratia¹⁹ reported finding two types of anthrax colonies similar to those described by Wagner. However using rabbits as the test animal he found the R form more virulent than the S form. Pesch,²⁰ in studying lytic-like areas occurring on agar slant cultures of *B. anthracis*, found, as a result of plating from such areas, colonies similar to those described by Wagner.

Haag²¹ has in the last year published work dealing directly with the so-called life cycle of *B. anthracis*. He has produced as a result of growing the organism in medium to which had been added its own filtrate or filtrates of decomposed matter, a wide variety of forms. These include asporogenic forms, cocci, motile as well as nonmotile rods, gram-positive and gram-negative forms. Also at one stage Haag describes a very small rod capable of passing a filter and virulent for mice. Haag is firmly convinced of the probability of a life cycle, involving possible forms of reproduction other than simple fission, as the basis for an explanation of these peculiar findings.

14a Centralbl. f. Bakteriöl., 1, O., 1914, 73, p. 81.

15. Compt. rend. Acad. d. Sc., 1914, 159, p. 340.

16. Centralbl. f. Bakteriöl., 1, O., 1917, 79, p. 425.

17. Ibid., 1920, 84, p. 386.

18. Ztschr. f. Hyg., 1922, 97, p. 12.

* For mice only.

19. Compt. rend. Soc. de biol., 1924, 90, p. 369.

20. Centralbl. f. Bakteriöl., 1, O., 1925, 93, p. 525.

21. Arch. f. Hyg., 1927, 98, p. 271.

A PRELIMINARY OBSERVATION

The first experiments performed, resulting in the isolation of the first variant of *B. anthracis* were quite incidental and had no part in a preconceived plan for the study of the dissociation phenomenon as related to *B. anthracis*. Having observed the interesting work of Soule²² in dissociating the hay bacillus, a casual interest arose as to the significance that such phenomena might have in a routine practice of using blood agar cultures of the anthrax bacillus in preference to plain agar cultures, in the routine inoculation of guinea-pigs for class use.

To this end a blood agar slant and a plain agar slant were inoculated from the stock culture of this organism. Plates streaked from these 24 hour slant cultures showed only the usual anthrax colonies. However on both plates some colonies appeared slightly more dense than others. From one of these more dense colonies (of the blood agar series) a tube of broth was inoculated. After 24 hours incubation at 37 C. an agar plate was streaked from the broth culture. On examining this plate, after incubation, an entirely different picture presented itself. There were two distinct types of colonies present. One was the usual flat anthrax colony, with the dull grey surface, giving a cut stone or cuneiform appearance by transmitted light. An examination of the border under the low power of the microscope revealed the classical Medusa head appearance. The other type of colony was raised and had a smoother structure than the normal anthrax colony. They lacked the cuneiform appearance of the latter when examined by transmitted light. Pure cultures of these two forms were injected into guinea-pigs. The animal that received the normal anthrax form died in 50 to 52 hours and a pure culture of the injected form was recovered at necropsy. In the case of the variant, the animal died 10 hours later and a pure culture of the variant was isolated from the dead animal.

Having obtained a killing culture which gave an atypical colony form corresponding to that described by Wagner, interest was aroused to explore further into the field of bacterial dissociation as applied to *B. anthracis*.

MATERIALS AND METHODS

Culture.—This work has involved primarily only one culture. This was brought by Dr. Novy in 1888 from Koch's laboratory and has been in constant use as a source of material for class study. It is a noteworthy fact that no irregularities have ever been noted with respect to colony formation.

22. J. Infect. Dis., 1928, 42, p. 93.

The ease with which the initial isolation of a variant was effected has not since been repeated. Although the first variant, as well as the other variants have been isolated many times from this stock culture, serious effort has been required for the production of variants. Hence the first isolation must be considered as a fortunate result.

The purity of the particular stock slant culture with which this work started was determined by removing a large representative sample and diluting it in broth. This was then immediately streaked to agar plates. This was repeated on two different days with the result that 55 plates in all were so streaked. About 200 colonies were present on each plate or a total of 11,000 colonies. No form other than the usual anthrax colony occurred in this large number of colonies.

Culture Mediums.—All mediums used, with the exception of desiccated differential mediums, where noted, were prepared from a base of cold infusion of lean ground beef in distilled water to which had been added 1 per cent bacto-peptone* and 0.5 per cent NaCl. For general plating purposes a 2 per cent agar with P_H of 7.7 to 7.8 was used. All P_H values given represent the values at the time the medium was actually used. After autoclaving, all culture mediums were incubated to test for sterility, before use.

Plate Readings.—The variation from one form of *B. anthracis* to another was determined by streaking agar plates from cultures to be examined for dissociation. After incubating such plates the number of each type of colony on the plate was estimated and recorded as percent of all colonies present.

Animal Inoculations.—Guinea-pigs weighing 225 to 350 grams and mice weighing 20 to 25 grams were used in virulence tests. The former were inoculated under the skin while an interperitoneal injection was found to be the method of choice in the case of the mice. All animals were inoculated with salt solution suspensions prepared from a 20 to 24 hour agar plate culture of the variant in question. Such suspensions were made up to a turbidity corresponding to a plate count of 100 million per cc. The inoculum was always plated following the inoculation to insure a definite knowledge as to the purity of the inoculum.

In the event of the death of the animal the time at which the animal was found dead was recorded and an autopsy performed. Gross pathologic changes were observed and agar plates were streaked from the spleen, liver and heart blood. (In later experiments dealing pri-

* Peptone from the same lot was used throughout the work.

marily with mice, it was found satisfactory to merely streak a bit of the liver onto an agar plate.)

INITIAL ISOLATION OF VARIANTS

As many authors reporting work dealing with microbic dissociation have used liquid mediums in preference to solid mediums for fostering this reaction, the early studies of the variant already described along with the normal culture form were made in such mediums. As a result of inoculating these two forms into plain broth and into broth to which had been added 10 per cent normal rabbit serum, variants with still different colony characteristics were obtained on plating from such cultures onto agar plates. By June 3rd, 1926, seven colony types of the anthrax bacillus had been obtained in the three months of study on the dissociation of this organism. The appearance of these colonies and the corresponding gelatin stab cultures is given in figure 1.

COLONY FORMS AND TERMS OF REFERENCE

I. Suggested Term R.*—The colonies on agar give a flat growth attaining a size of 3 to 5 mm. after 24 hours incubation and are characterized by a dull, grey surface. When viewed by transmitted light these colonies give a beautiful cuneiform or cut glass appearance. This is particularly characteristic of colonies 12 to 20 hours old. Examination under the low power of the microscope reveals the classical Medusa head appearance of the edge of the colony. The internal structure under the low power appears wavy. To the touch of a platinum wire the colony is friable.

To this so-called normal culture type the term R is suggested out of deference for its rough, flat colonies.

II. Suggested Term RS.—The organisms of this culture type give rise to a colony which, with respect to its colony characteristics is intermediate between the R and S forms. The colony is raised and often shows a somewhat smooth surface by reflected light. However by transmitted light it is somewhat rough. Examination under the low power of the microscope reveals a somewhat wavy edge but free from the wavy isolated single or grouped filaments that make for the Medusa appearance of the R form. The internal portion of the colony is opaque

* The Roman numeral refers to the temporary term of reference used in the preliminary report.²³

23. Proc. Soc. Exper. Biol. & Med., 1927, 24, p. 959.

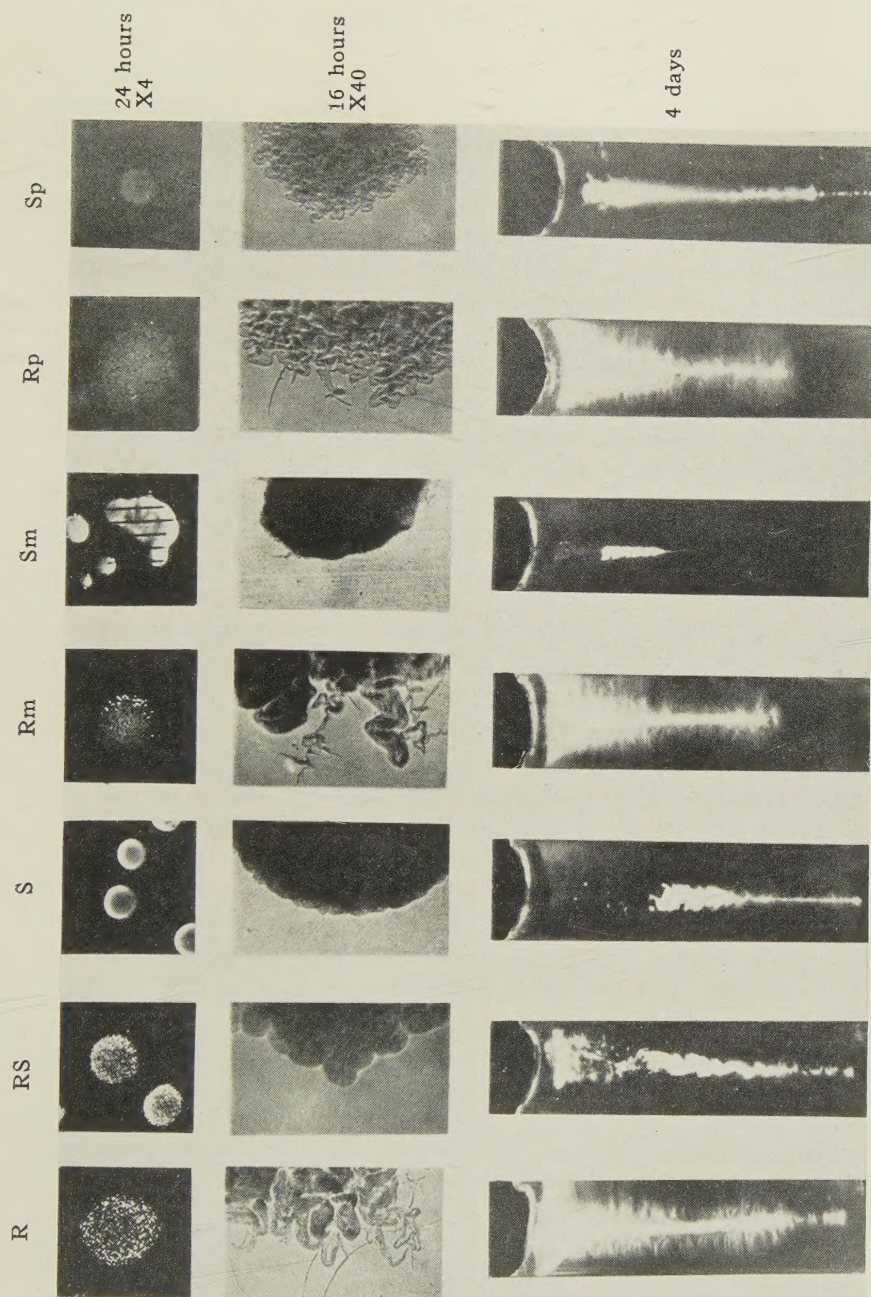


Fig. 1.—Colony structure of the variants on agar and growth in gelatin.

to transmitted light in microscopic examinations. The colonies range in size from 3 to 4 mm. at the end of the usual 24-hour incubation period.

The term RS is suggested for this form since its properties appear to be intermediate between the R and S forms. An even more significant reason is that in the change of R to S or vice versa, this form appears. In this sense it seems to be a true intermediate form. As will later be pointed out, all of this work tends to indicate a gradual rather than a sudden change in passing from one form to another. Hence the culture just described represents an arbitrary choice of one such intermediate occurring in the dissociation of $R \rightleftharpoons S$.*

This form agrees very closely as far as colony properties are concerned with the B type described by Wagner.¹⁷ Many of the earlier colony variations described for the anthrax bacillus belong to this general colony type. In the first reports of Preisz,¹⁰ as well as in his later reports,^{11,5} this form has been described. Likewise Markoff,¹² Eisenberg¹⁴ and Hess²⁶ have reported colony forms similar to this one. In some of these cases the described form may have had more of the R properties. This is probably true of Eisenberg's sporogenic strains.

III. Suggested Term S.—Colonies of this culture type are characterized by the smoothness of surface and border. This raised colony (1½ to 3 mm.) presents a glistening appearance when viewed by reflected light and has a cream color. By transmitted light it appears homogeneously opaque. Examination under the low power of the microscope reveals a freedom from the medusa appearance of the R form or the wavy appearance of the RS form. The opaque center structure extends to the very edge of the colony. This colony is much softer in texture than the R colony and could not be described as friable. It is the cultural characteristics of this form, particularly its colony structure, that have prompted the designation of this type as the S form.

It is difficult to determine from descriptions whether the variants of *B. anthracis* that have been described in the past belong to the S or the RS form as described here. However, it is probable that both Preisz⁵ and Markoff¹² have described this form and possibly Baerthlein¹³ has also reported its occurrence.

* Although Neisser⁶ and DeKruif² have emphasized the abrupt nature of such changes, many other workers have noted a more gradual change. Arkwright¹ and White²⁴ have both emphasized this point, and Toennissen²⁵ has reported the intermediate degrees of encapsulation in the Friedländer bacillus.

24. Special Rep. 91, Med. Res. Council, 1925.

25. Centralbl. f. Bakteriöl., 1, O., 1914, 73, p. 241.

26. Arch. f. Hyg., 1920, 89, p. 237.

IV. *Suggested Term Rm.*—The form of colony to which it is suggested to apply the term Rm resembles the colony characteristics of the R colony in certain respects. It has the characteristic cuneiform structure and the usual Medusa appearance of the colony edge. However, the colony is not flat and dull but raised and glistening. In addition it possesses the distinguishing characteristic of having a mucoid texture. When touched with a wire, which is then slowly raised, strings of the culture 2 to 3 cm. long may be pulled up. Because of the mucoid property associated with otherwise rough characteristics the term Rm is suggested. This colony form has probably been described by Preisz⁵ and Markoff.¹²

V. *Suggested Term Sm.*—Colonies 16 to 20 hours old of this form, if well isolated, resemble to a great extent the S colonies. They have similar smooth appearances of surface and border. However, in event two such colonies are adjacent they readily "flow" together. This tendency increases rapidly with age. By the end of 24 hours incubation, the colonies become semitransparent so that the point of a pen can be readily distinguished when placed beneath the colony. In a fairly heavy streak on an agar plate with colonies somewhat crowded, the streak soon (20 to 24 hours) appears as a homogeneous mass of highly mucoid material with the identity of the individual colonies completely lost. The result of touching such a growth with the needle and drawing slowly away is striking, since threads even 20 to 30 cm. in length can be drawn out. A microscopic examination of the border of a 16-hour colony under the low power shows the same general picture as a similar examination of the S form. However, as the colony ages (24 to 30 hours) the structure increases in transparency. The organisms near the edge of the colony become more widely separated from each other and this separation increases with the age of the colony.

It appears that the general smooth characteristics of the S form are modified by the addition of a mucoid characteristic. Hence the term of reference Sm is suggested. The reports of Preisz,⁵ Markoff¹² and Baerthlein,¹³ deal with a variant of the anthrax bacillus similar to the one described here.

VI. *Suggested Term Rp.*—Soule²² has described a third form of variant of the Hay bacillus and has designated it as a phantom culture because of the very thin, film-like growth occurring on plain agar. An analogous type of growth was independently found in the case of the anthrax bacillus. Further it was found that it was possible to dis-

tinguish two forms of "phantoms." The first form has been designated as Rp while the second has been termed Sp. The growth of the Rp form is very meager on plain agar and appears as a film. The colonies are about the size of those of the R form and have a border quite similar to the latter. However, the colony never rises to more than a mere film in depth and often a plate with a luxuriant growth of this form may appear as a sterile plate until examined more closely. The term Rp is suggested for this type of growth for the reason that it appears to partake of both the R characteristic as well as the phantom characteristic.

This general type of colony may have been observed by Scagliosi²⁷ in the culture recovered from a silk thread which initially had been soaked in spore material and allowed to dry. Cultures were made from the thread after 10 years. Scagliosi found a general weakening of the biological properties of this recovered strain. It grew slowly in broth. On agar it grew with a thin grey-white growth and liquefied gelatin slowly. The organisms were somewhat shorter and thinner than the ordinary *B. anthracis*. The culture killed guinea-pigs.

VII. Suggested Term Sp.—The colonies of this type are more the size of the S colony. The growth, although quite limited on plain agar, is somewhat heavier than the Rp. The border of this colony is not as free of Medusa appearance as the S form, but at least this tendency is much more limited than it is in the Rp. It is possible that a phantom form with truer S properties may be found in the future. The reason for suggesting the terms of reference Sp for this form is analogous to that for suggesting the term Rp, namely: that this form appears to have the dual characteristics of smoothness and film-like growth.

Intermediate Forms.—Many intermediates have been met with in this work and their occurrence has been designated by making use of the following scheme. In the event of an intermediate between R and S, of course the RS form represents one such form. If the colony appears to have more of the X property, however, that letter in the term RS is underscored, RS. Or if the S properties predominate the term becomes RS. In the description (by terms) of other intermediates a somewhat more bulky system has been used. For example, if the colony appears to have its properties divided between the R and Rm forms it is expressed R-Rm, R-Rm or R-Rm, depending upon the extent that the

27. Centralbl. f. Bakteriol., 1, O., 1904, 37, p. 649.

muroid characteristic is associated with the rough property of the form. Similarly an expression as S-Sm would indicate a smooth colony with an intermediate grade of the muroid property. The use of such a symbolic system for describing quite varied colony forms simplifies the great detail which would otherwise work a considerable handicap on the recording and presentation of data such as are involved in work of this nature.

Isolation of Variants.—The experiments resulting in the initial isolation of the variants are not as significant in general as later experiments when the appearance of these strange colony forms were looked for and were hence more readily recognized. Nevertheless it seems desirable to present briefly the origin of these seven variants.

R Form.—This being the colony type characteristic of the original culture, the variant stock of this form had its origin in an isolated typical anthrax colony of the initial experiment described in the introduction.

S Form.—For a short time the original atypical colony form was carried as the S form. It soon became evident that colonies of a smoother structure were appearing, hence this original culture was abandoned and a new and smoother colony selected in the following manner:

On a plate streaked from a 1% peptone solution culture of the so-called S form after 18 days incubation, three types of colonies were noted, namely the R, RS and S. From one of the smoothest of these S colonies a subculture to an agar plate was made which has since been carried as the S form.

RS Form.—After the selection of a true S colony form it was decided to carry a form intermediate between the R and S with the designation of RS as already noted. A plate inoculated from an 18-day old culture of the early so-called S form in a medium composed of broth 1 part and filtrate* of Sm 1 part, gave three types of colonies R, RS and S. The selection of the RS for the variant stock was made from one of the RS colonies on this plate.

Sm Form.—The S form was inoculated into 10 cc. of a 10% mixture of normal rabbit serum in broth. The plate inoculated on the fourth day from this culture showed besides the regular so-called S form which had occurred on previous plates, colony types now designated as the Sm form.

Rm Form.—A flask containing 300 cc. of a 10% normal rabbit serum in broth was inoculated with the R form. During the first 21 days of incubation three colony forms had made their appearance, namely the R, S and Sm on plates streaked from this culture. On the 9th day of the experiment the streaked plate showed R and Sm culture types. The next plating on the 21st day showed R, Sm and Rm forms.

Rp Form.—The R form was inoculated into 25% normal rabbit serum in broth. Plates streaked at the beginning of the test as well as after the second day showed only R forms. However, plates streaked on the third day from this culture showed besides the usual anthrax colony a nearly invisible growth which has come to be designated as the Rp type.

Sp Form.—From the same culture that the Rp type was isolated a plate was streaked on the fifth day. On this plate appeared besides R and Rp colonies,

* A 24-hour culture of Sm on an agar plate was taken up in broth and filtered through a Berkefeld N. filter.

also S colonies and phantom colonies more limited and S-like in their contour than the Rp form. This has been designated as the Sp form.

This culture has been replaced in the stock of variants because it appeared to be shifting to an Rp type of colony. A more typical Sp colony was selected from a plate streaked from a 7-day culture of Sp in 10% serum broth to replace the former culture.

In brief this is the history of the initial isolations of the variants of *B. anthracis* with which this paper deals. Cultures of these forms have been carried along continuously as will be described.

PROPERTIES

Growth in Broth.—The definite results reported for the growth characteristics in broth of the R and S forms of many other organisms are somewhat lacking in the case of *B. anthracis*. It can be stated that the S form grows with a limited amount of clouding of the upper portion of a tube of broth, but at the same time it does settle to the bottom rather rapidly, forming a more or less granular deposit. The R form gives the usual clear supernatant liquid with a cottony mass at the bottom. The RS as a rule may tend to give an intermediate type of growth but because of the slight difference between the R and S growth in broth no point is made of this. The Rm and Rp, and the Sm and Sp follow the tendencies of the R and S forms respectively. This failure to get definite results on the growth in broth is not at all surprising if we consider the size of the organism and the lack of motility of all forms.

Salt Solution Suspensions.—When the ease with which salt solution suspensions of these forms can be made, is used as the criterion for differentiating the R from the S (Arkwright¹), definite and constant results follow.

The R form breaks up in .85% salt solution with difficulty. The resulting suspensions are seldom smooth and rapidly settle out. This is, of course, the usual observed difficulty and has been a contributing factor in the difficulties besetting agglutination tests with this organism (Noble²⁸).

The RS form is intermediate between the R and S with respect to its behavior in salt solution. It approximates more closely the behavior of the S form.

The S form is readily put into suspension in salt solution giving a relatively smooth suspension.

The Rm form, although presenting less difficulty to the formation of a salt solution suspension than the R form, nevertheless gives more trouble than the RS or S forms. It gives a smoother suspension than the R form.

The Sm form breaks up in a very characteristic manner. Strands of a slimy nature follow the needle, as it is rotated in the salt solution. The culture slowly breaks up, giving a smooth suspension.

28. J. Immunol., 1919, 4, p. 105.

The phantom cultures (Rp and Sp) break up in salt solution very readily but give a rather granular suspension which is more smooth than that given by the R form, but not as smooth as the suspensions of the other forms.

Growth on Agar.—The growth of the variants on agar as observed after the usual period of incubation has already been described. If a point inoculation of the variants is made in the center of separate plates of agar, however, and the incubation of the colonies continued under conditions which prevent drying of the medium, very interesting results are often obtained. As the colonies increased in age many peculiar formations developed. These will be reported in a later paper with Mrs. Parker.

Morphology.—Before describing the morphology of the variants, attention is called to figure 2. From this figure the essential differences in the morphology of the variants is to be noted. Also changes in the morphology of the various forms with increase in age of the culture examined, are noted.

R Form.—The organisms of this form were of the usual cylindrical shape ranging in length from 1.5 to 4.5μ with an average length of 3.1μ . The width of these cells averaged slightly less than 1μ ($.97\mu$). Chains of considerable length were frequently observed in this form, particularly if the examination was made from broth. As the culture aged, spores appeared, occupying the usual median position when seen within the cell. No motility was ever observed in this form, or in any of the other forms described in this paper.

RS Form.—The point of difference between this form and the usual anthrax type was in the formation of chains. Only short chains were observed in this type. The cells were frequently slightly curved or vibrio shaped when cultures of 24 hours or older were examined. Spores were produced by this form.

S Form.—The rods of this form were shorter and heavier than those of the R form (average 2.5μ long and 1.0μ wide). The cells were frequently vibrio shaped and usually occurred as pairs or very short chains. Examinations made from agar cultures (24 hours or older), showed a marked tendency for the rods to shorten and widen. Thus they often resembled small yeast cells: Spores were formed by this culture.

Rm Form.—With the exception of spore formation the morphology of this form is similar to the R form. Although spores have been observed in this form they occur much less frequently than in the R form.

Sm Form.—The general shape of the rods of the Sm form in young cultures was similar to the S form, but examinations made of agar cultures (24 hours or older), showed a somewhat less tendency to form the coccoid bodies than did the S form.

Stained preparations of smears made from water or saline suspensions of old (48 hour) agar cultures of this form were characterized by a background of weakly staining material (gram-negative). The organisms themselves stained deeply by the Gram method. Spores were not frequently observed in this form.

Rp Form.—The morphological characteristics of young (12 hour) agar cultures of this form do not vary greatly from the R form. Examination of older cultures of this form revealed very characteristic swellings. These swollen or invo-

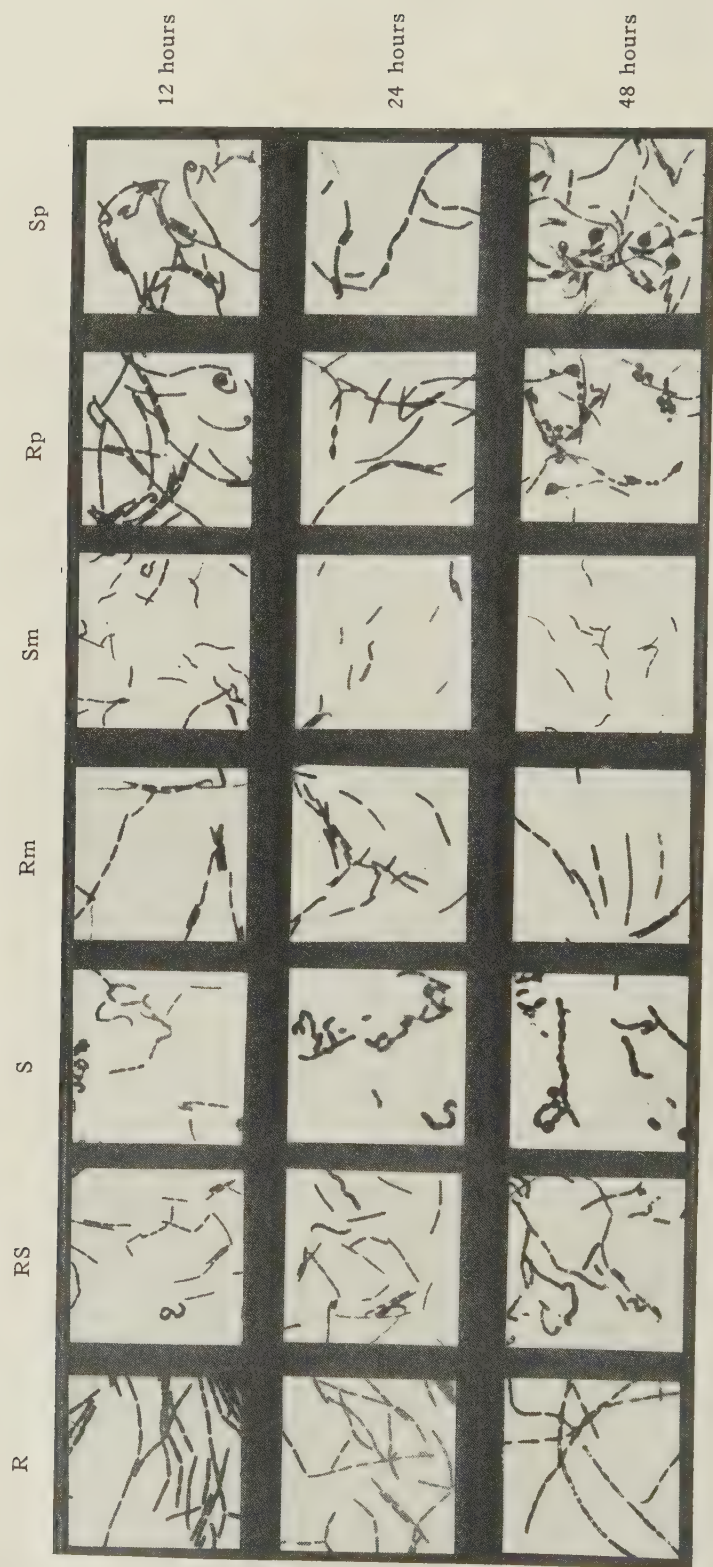


Fig. 2.—Morphology of variants, and changes in morphology of individual variants with increase of age of culture. $\times 800$.

luted forms appeared with absolute regularity in agar cultures of 48 hours or older. Young subcultures to fresh medium revealed only the more typical anthrax form. It is impossible with the facts at hand to attribute any significance, with respect to a special reproduction process (Mellon²⁹) to such forms. The coincidence of the occurrence of these odd morphological forms with the aging of the culture, coupled with negative filtration, and warm stage observations, warrant the tentative interpretation "involution forms" to be applied to these peculiar cell types.

Spores were formed in very large numbers in this form during the first year of this investigation. At the present time, however, this form is practically asporogenic.

Sp Form.—This form agreed in general, morphologically, with the Rp form. The difference existing between the R and S and the Rm and Sm forms did not manifest themselves as markedly in the case of the phantoms as in the other forms. Shorter chains were present in the Sp than in the Rp form. This was the chief point of difference between these forms. Spore formation has practically disappeared in this form as it has in the Rp form.

Staining.—All forms stained readily with the anilin stains and retained the stain when treated by the Gram method. For the purpose of accurate comparison all forms, properly marked, were stained on a common slide. There was, however, a quantitative difference in the number of cells of the various forms which retained the dye when the length of staining and of decolorizing in the Gram method were altered so as to bring out these differences. The mucoid forms took an intense stain while the R, RS and S forms stained with a somewhat less intensity. In all of these forms 95 to 100% of the cells were gram-positive. In the case of the phantom forms 40 to 50% of the cells lost the stain while the remaining cells stained with an intensity comparable to those of the R form.

Biochemical Reactions.—The effect of cultures on various mediums is as follows.

Gelatin.—The classical inverted fir tree type of growth resulted following a stab inoculation in 12% gelatin in the case of the R, Rm and Rp forms. The RS and Sp types gave an abbreviated tendency toward this characteristic while the S and Sm growth in gelatin entirely lacked this characteristic.

Liquefaction of the gelatin stabbed with the R culture usually began after four to five days as indicated by the cupped depression formed at the top of the stab and partially filled with liquefied gelatin. This deepened and widened until after a few days a layer of liquefied gelatin covered the top. Liquefaction in the case of the other forms proceeded in general along similar lines. In the case of the S and Sm forms the liquefaction cup became quite deep before the liquefaction at the top had reached the edge of the gelatin. This was probably due to the arborescent nature of the first growth of the R as contrasted to the S form. As to the speed of liquefaction by the various forms it can be said in general that the R form of *B. anthracis* is the most active in this respect followed closely

29. J. Bact., 1925, 10, p. 481.

by the RS and Rm forms. The S, Sm, Rp and Sp types have a somewhat slower liquefying ability.

Litmus Milk.—Neutralized fresh skimmed milk to which litmus solution was added was used as well as a commercial desicated "purple milk" in determining the growth characteristic of the variants in this medium. Acid was produced by all forms between 36 and 72 hours following inoculation. Considerable curd production was noted in the R cultures at 72 hours with some less in the case of the other cultures. Liquefaction of the curd began in the case of the R, RS and S cultures at 136 hours and somewhat later in the cases of the other forms. In general the point can be made that the R form has increased activity over the other forms with respect to the curdling of milk and subsequent liquefaction of the curd. These results are in accord with Gamaleia⁹ who, in 1888, reported the "attenuated" vaccine cultures had slower coagulating and proteolytic activity than the normal anthrax cultures.

Russell's Double Sugar.—Only slight quantitative differences in acid production were observed when the various forms were grown on Russell's double sugar. (Digestive Ferments Co.). The phantom forms were a little slower in acid production than the other forms. No gas was produced in any case.

Blood Agar Plates.—Jarmi³⁰ in 1913 reported more rapid hemolysis of blood by anthracoides strains and *B. mesentericus* than by the anthrax bacillus. This work suggested the possible application for distinguishing these organisms from *B. anthracis*. This has recently been supported by the work of Wagner³¹ (1923) and Hallerman³² (1925).

Hence it was of interest to inoculate these various forms onto blood agar plates, by the point inoculation method, along with the hay bacillus. At 18 hours a definitely marked zone of hemolysis surrounded the subtilis colony while the anthrax variants were free from any such tendency. The results remained essentially the same until the 36 hour reading. At that time a small diffuse zone of hemolysis appeared around the R colony. Some slight hemolysis appeared in other colonies after 42 hours.

A point of considerable interest is the excellent growth of the phantom colonies on blood agar. On this medium these cultures grow as luxuriantly as the R form. On subculturing back to plain agar, however, the phantom growths were again obtained. Is it possible that the phantom cultures are cultures with a limited food assimilating mechanism requiring the special diet furnished by blood for its full development?

Milk Agar Plates.—Wagner³⁷ reported that the usual anthrax culture had greater proteolytic action on milk, as determined by milk agar plates, than did the variant B which he isolated from a human case of anthrax. When such plates (agar to which 10% sterile skimmed milk had been added before pouring) were incubated after inoculation with the various forms, it was noted that the milk was acted upon slowly in all cases and this action was observable after four or five days. *B. subtilis* inoculated on such plates gave a definite zone of clearing after 18 hours incubation.

Potato.—All forms gave a white growth on potato which changed to a light grey after 120 hours in the case of the R. This slight discoloration was less marked in the other forms. The growth of the phantom forms was very poor on potato slants.

Indol Production.—Tests for indol made on cultures of the various forms grown on tryptophane broth were negative. Similar control tests performed on

30. Centralbl. f. Bakteriöl., 1, O., 1913, 70, p. 72.

31. Ibid., 1923, 90, p. 433.

32. Ibid., 1925, 96, p. 419.

cultures of *B. coli* grown in the same medium gave definite positive results, indicating the satisfactory nature of the medium and reagents (Ehrlich's and a saturated solution of potassium persulphate) for demonstrating indol when actually present.

Hydrogen Sulphide Production.—Cultures of the variants on Kligler's lead acetate medium showed no blackening. Control cultures of both *B. coli* and *B. paratyphosus* *B.* readily darkened the medium.

Respiratory Quotients of the Variants on Plain Agar.—It was decided to make a limited metabolism study of the variants, grown on plain nutrient agar, including such items as the changes in the manometric readings as a result of growth in a closed system, base production as manifested by CO_2 fixation, and respiratory quotients.

The details of this type of study are so well developed in the work of Novy, Roehm and Soule,³³ that no effort will be made to repeat a description of the general details here. In the selection of the general type of experiment two plans have been used by these workers. The first is to grow the organism on agar slants in tubes of small volume (100 cc.) connected to a compensating manometer. The second method is to place agar slants, freshly inoculated, in Novy jars of varying capacity in the neighborhood of 1500 cc. and these in turn are connected to manometers. The second method has certain advantages in determining accurate respiratory quotients. The volume is larger and enables one to draw extra samples of gas for duplicate analyses. Again analysis may be made from time to time during the progress of the experiment without seriously interfering with the experiment. A third and very important advantage of the jar method over the tube method is in minimizing the error of extracellular carboxylase activity in the case of certain organisms. This has been clearly pointed out by Novy and coworkers in the case of *B. Subtilis*. They have shown that when the respiratory quotient, as determined by the tube method, is recalculated on the basis of the jar method, the results for the respiratory quotient of this organism on plain agar approaches more closely the value to be expected in metabolism utilizing protein as a source of energy.

However, the tube method is not without advantages. Because of the smaller volume, the manometric changes are more marked than in the jar method. This, in the case at hand, seemed a very desirable advantage in order to note differences in the growth curves of the various forms. A second advantage of some less significance is the greater economy of space obtainable with the tube method over the jar method. In this work 14 tubes of cultures with attached manometers as well as

33. J. Infect. Dis., 1925, 36, p. 109.

two medium control tubes and manometers were set up at a given time. It would not have been convenient to run as many jars in a given experiment because of the limitation of incubator space.

The tube method appeared, for the work at hand, to be the method of choice. Since Novy, Roehm and Soule have pointed out the objection (solubility of CO_2 in rubber) of rubber stoppers in exact gas studies, only glass capped h-tubes were used in this work. Only details especially observed or related to this problem will be given here. The determination of the volume of the h-tubes, calibration of manometers, gas analysis and carrying out of respiratory quotient determinations were conducted according to the technic described in the paper referred to. Certain special precautions were observed:

One batch of medium was used for all experiments of this type.

All h-tubes, manometers, etc., were placed in the hot-room over night to insure early equilibration with respect to temperature and moisture.

All tubes were inoculated in the hot-room and before connecting the capped tubes with the manometers special emphasis was given to the precaution of injecting steam into the glass caps.

All tubes were inoculated from agar plate cultures of the variant involved after a careful examination indicated apparent purity of the variant.

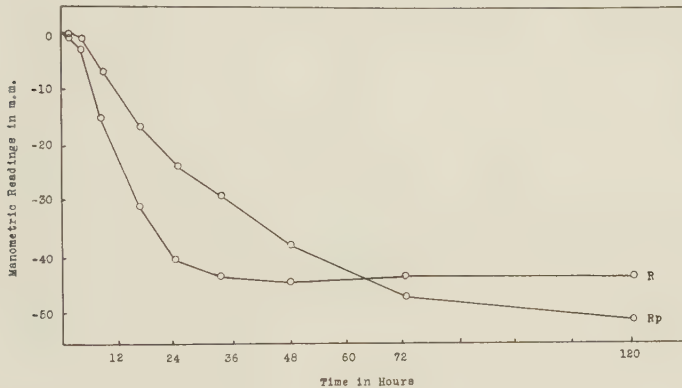
An examination of the culture present at the end of a test was made, prior to the determination of the dissolved CO_2 . This examination included macroscopic observations of the growth in the tube and subcultures to agar plates. Results indicating dissociation during the course of the experiment are recorded in table 2.

Results.—The development of the negative pressure results from two causes. The first is the unequal return of CO_2 , when protein is utilized for energy, for the O_2 taken up by the organism. This factor is indicated by the respiratory quotient $\frac{\text{CO}_2}{\text{O}_2}$. The theoretical respiratory quotient can be readily deduced for substance of known composition from the chemical equation for the oxidation of these substances to CO_2 and H_2O . Novy and coworkers have done this and found experimental checks when microorganisms were grown on mediums containing glycerol or glucose. A second factor in the production of this negative pressure is the fixing of CO_2 in the medium due to CO_2 combining power of the medium itself or to alkaline substances formed in the proteolytic activity of the organism in its growth. Hence charting the development of negative manometric pressure (when the organism is utilizing protein or glycerol), gives in a sense a measure of the metabolic activity of the organism.

In the routine used, it was a practice to set up seven inoculated tubes with attached manometers for a given variant. Readings of the mano-

meter were made at frequent intervals throughout the experiment. At the end of each 8, 16, 24, 32, 48, 72 and 120 hours one tube was removed for a gas analysis and the determination of the dissolved CO_2 in the medium. The average of the manometric readings of seven tubes for the 2, 4 and 8 hour intervals, 6 tubes in the case of the 16 hour interval, 5 for the 24 hour, 4 for the 32 hour, 3 for the 48 hour, 2 for the 72 hour and one for the 120 hour, were thus used in plotting curves for the various forms. It is thus evident that the greatest accuracy of the curves is in the first 32 hours.

Such curves were practically identical for all forms except the phantom types. The curves for the R and Rp forms are seen in chart 1. They are typical of the curves obtained for the RS, S, Rm and Sm, and



The development of negative pressure in a 100 cc. h-tube as the result of growth of the R and Rp forms on plain agar.

Sp forms respectively. Note the short latent period in the R curve followed by a rapid drop in manometric reading. This drop terminates at a time corresponding to an approximating zero oxygen tension as seen in table 1. The Rp form has a longer latent period and a less sudden drop in manometric readings.

The dissolved CO_2 value (indicative of protein decomposition) rises until a value of 5 to 6 cc. of dissolved CO_2 per 10 cc. slant is obtained in the case of all forms except the phantoms. These cultures do not fully attain this value even after 408 hours.

The value for the corrected real respiratory quotients (table 1), are all similar and apparently too high for an organism utilizing protein as a source of energy. However, this apparent high value is probably due to the extracellular carboxylase activity.

TABLE 1.—ANALYSIS AND QUOTIENTS OF VARIANTS OF B. ANTHRACIS GROWN ON PLAIN AGAR IN GLASS-CAPPED, 100 CC. H-TUBES

Variant	Hours	Manometric Reading, Mm.		Corrected Analysis, %		Dissolved CO ₂ Cc.	Corrected Real-Respiratory Quotient
		Observed	Calculated Real	CO ₂	O ₂		
R	8	—16.8	—19.1	5.32	12.99	2.58	1.030
	16	31.1	37.0	10.10	5.57	3.53	.911
	24	44.6	51.8	13.02	0.57	5.25	.935
	32	42.3	56.2	12.89	0.09	5.53	.916
	48	44.4	51.7	13.64	0.00	5.47	.944
	72	41.5	48.4	14.12	0.00	6.07	.994
	120	—42.7	—47.2	14.27	0.00	5.80	.989
					Average	4.89	.940
RS	8	—12.8	—19.6	4.64	13.59	2.03	.942
	16	29.7	36.8	9.40	6.37	4.03	.970
	24	38.6	40.7	10.52	4.70	4.97	.980
	32	46.3	50.8	13.76	0.00	4.65	.910
	48	41.6	48.5	14.07	0.00	5.43	.954
	72	39.6	49.0	14.03	0.00	5.07	.944
	120	—41.5	—48.5	14.09	0.00	5.43	.983
					Average	4.52	.955
S	8	—11.0	—13.6	2.87	15.97	1.95	1.057
	16	32.3	37.3	7.55	7.91	4.45	.999
	24	47.5	51.0	11.78	1.72	5.50	.947
	32	50.8	56.4	12.76	0.00	6.11	.954
	48	51.6	54.5	13.01	0.00	6.11	.956
	72	49.6	61.2	11.95	0.10	6.35	.937
	120	—47.4	—52.5	13.28	0.00	6.22	.987
					Average	5.24	.977
Rm	8	—20.3	—22.2	4.40	13.22	2.56	.957
	16	35.5	38.1	8.40	6.95	3.67	.914
	24	40.5	44.6	10.73	3.72	5.18	.938
	32	46.5	51.5	12.54	0.83	6.35	.994
	48	48.4	50.0	13.20	0.39	6.25	.982
	72	46.5	51.4	13.38	0.00	5.80	.959
	120	—50.6	—52.8	13.19	0.00	5.55	.937
					Average	5.05	.954
Sm	8	—12.1	—13.2	2.25	16.83	1.39	.934
	16	29.3	26.7	5.71	11.45	3.45	1.020
	24	38.4	38.2	8.14	7.40	4.45	.974
	32	42.4	44.8	10.49	4.09	4.89	.951
	48	48.6	53.4	13.06	0.30	6.02	.968
	72	51.8	53.0	13.45	0.00	4.49	.886
	408	—48.6	—55.8	13.04	0.00	5.12	.901
					Average	4.26	.948
Rp	8	— 8.1	—11.5	1.55	17.59	.94	.801
	16	14.2	17.7	2.44	15.81	1.77	.885
	24	23.2	26.4	5.20	11.75	2.16	.858
	32	25.4	25.4	5.16	12.18	2.77	.926
	48	35.3	29.2	8.08	8.73	2.99	.940
	192	49.4	45.5	13.26	0.00	4.89	.907
	288	—43.6	—48.5	13.83	0.00	4.49	.918
					Average	2.86	.891
Sp	8	— 8.1	— 8.1	1.42	18.20	0.95	.977
	16	11.1	15.7	2.49	16.05	2.22	1.063
	24	19.3	23.7	4.27	13.12	3.44	1.072
	32	Analysis sheet lost					
	48	20.3	26.8	5.50	11.43	2.94	.950
	192	45.1	50.1	13.06	0.48	4.55	.877
	288	—40.4	—46.2	13.16	0.00	4.55	.886
					Average	3.11	.971

From these studies no striking qualitative difference between the variants manifests itself. Quantitatively all forms react similarly with respect to proteolytic activity, as manifested by fixation of CO_2 , and development of negative manometric pressure except the phantom forms. They have a retarded activity with respect to both of these functions.

TABLE 2.—OBSERVED DISSOCIATION IN SUBCULTURES FROM THE H-TUBE CULTURES USED IN RESPIRATORY QUOTIENT DETERMINATIONS

Form Inoculated	Tube-Hours	Subcultures		Appearance of Slant
			%	
Sm	24	Sm	20	
		S	80	
	32	Sm	20	
		S	80	
	48	Sm	5	
		S	95	
	72	Sm	5	
		S	95	
	408	Sm	30	
		S	40	
		RS	30	
Rp	8	Rp	75	Phantom growth
		R	25	
	16	Rp	95	Phantom growth
		RS	5	
	24	Rp	50	3 RS colonies *
		R	50	
	192	Rp	50	2 RS colonies * †
		S	50	
	288	Rp	50	12 RS colonies *
		RS	50	
Sp	8	Sp	25	Phantom growth
		S	75	
	16	Sp	15	50 RS colonies *
		S	85	
	24	Sp	50	200 RS colonies *
		S	50	
	48	Sp	50	2 RS colonies * †
		S	50	

* It is impossible to clearly differentiate colony structure in secondary colonies arising from phantom growths. Hence the term RS has only the significance that it indicates a colony of heavier growth than the phantom and appears to be similar to the RS form. The subculture is a more certain index of the nature of the secondary colonies.

† Eroded areas, noted on these slants, resembled lytic areas, but were non-transmissible (fig. 3).

Dissociation has been observed in the agar slant cultures used in these experiments (table 2). The effect that this dissociation had on the results is manifested in a few aspects of the data obtained. From table 2 it is noted that the 24-hour tube of Sp had a marked secondary growth of S (200 colonies). Referring to table 1 shows that more CO_2 was fixed in the 24-hour tube than in the 48-hour tube. This can probably be attributed to the increased activity of the S over the Sp form in fixing CO_2 .

Peculiar nontransmissible, eroded areas were noted in the 48-hour Sp tube and the 192-hour Rp tube. The former is shown in figure 3. No significance can be attached to these findings from the data at hand.

MAINTENANCE OF STOCK

Since the original isolations of these variants, dating back ultimately to the R form, they have been carried on agar plates entirely. This procedure has extended over two years, with transplants being made every two or three days. The majority of such transplants have been from isolated colonies. Because of the large number of serial single colony isolations, objection to the failure to use the so-called single cell isolation technic seems unwarranted. Dissociation of these forms "purified" by a year and a half of such serial transfers have given rise to variants as readily as the earlier cultures. This tends to discount the objection of a mixed culture.

To give the detailed history of the stock variants would be entirely too time consuming and so the discussion on this phase of the work will be limited to certain necessary considerations. In general it may be said that the various forms have remained remarkably true to type. The R form has never shown colonies other than of the R type. The S form has at times given colonies tending toward the RS in properties but by one or two colony selections of the more typical S colony forms the culture soon was within the limits set for this type. The RS form has tended to the S form of colony at times and less frequently to the R, but by methods of selection these tendencies to change have been corrected. The phantom forms have been exceedingly stable in their properties. At times, however, they have given rise to R or RS forms. The maintenance of the mucoid colony forms in the early part of this study gave rise to considerable difficulty and led to a series of experiments which will be reported somewhat in detail.

EFFECT OF MEDIUM ON COLONY PROPERTIES OF THE MUCOID VARIANTS *

The early efforts to maintain cultures of the Sm variant were beset with difficulties. Often the isolated Sm colonies were opaque and entirely lacked the mucoid characteristics of this type. It was early noticed, however, that the heavy streaks were typical of the culture while the isolated colonies were not. This led to the following experiment.

* In presenting these experiments attention is called to but one experiment of a kind, although in every case this was amply repeated to make certain of the results.

Exper. M. A. 1.—An inoculation along the design of a ring, 2 cm. in diameter was made with the Sm culture. Then a point inoculation in the center of the ring with the same culture was made. A control point inoculation unrung, of the Sm culture was made on the other half of the plate.

The results from this experiment were definite. The unrung, point inoculation had given rise to an opaque, nonmucoid colony while the rung colony surrounded by the growth of Sm was perfectly mucoid and semitransparent. This experiment was repeated using a ring of growth of the R form of *B. anthracis*, as well as of *B. subtilis* and *B. prodigiosus*. The results were the same. Apparently there were some products produced by *B. anthracis*, Sm and R forms at least, and also produced by *B. prodigiosus* and the hay bacillus, which exerted a favorable influence in preserving the mucoid characteristic of the Sm form. A knowledge of the nature of this product seemed desirable, as it might enable one to maintain this very striking colony type. As there was no contact between the outside growth and the centrally inoculated colony, it seemed obvious that the product had either diffused through the agar or, as a gas, had been absorbed by the colony or the agar on which the latter was growing. Experiment M. A. 4 gives evidence on this point.

Exper. M. A. 4.—With the aid of a sterile cork borer, two concentric rings 12 mm. and 18 mm. were cut in a hardened agar plate and the included zone of agar was then removed aseptically. A culture of *B. prodigiosus* was inoculated on the outside of the "moat" while in the center of the "island" the Sm culture was point inoculated. Controls included a similar "island" without *prodigiosus* as well as a repeat of the set up for experiment M. A. 1 with *prodigiosus* as the culture inoculated along the design of the ring.

The results from this experiment indicated that the "moat" apparently made no difference in the favoring activity of the *B. prodigiosus* growth on the development of the typical colony. The unrung controls were devoid of the mucoid characteristic. Apparently, the favoring influence of the associated culture exerted its effect through a volatile product of growth which was absorbed by the culture or by the medium. However, to check this idea further another type of experiment was planned.

Exper. M. A. 7.—Four petri dishes were poured with agar and after cooling, two were inoculated by point inoculations with the Sm culture. The third dish was inoculated over the entire surface with *B. prodigiosus* and was then inverted over one of the Sm point inoculated plates and held in place by strips of adhesive tape. The fourth or sterile plate was inverted over the second Sm plate which served as a control.

After 24 hours incubation the results from this experiment were very definite and showed the favoring influence of a distant growth of *B. prodigiosus* on the development of the mucoid properties of the Sm culture. The control colony was lacking in the mucoid characteristic. The next step was to determine whether the effect in question was upon the culture directly or indirectly through intermediate action on the medium. Experiment M. A. 11. gives evidence on this question.

Exper. M. A. 11.—A plate inoculated with *prodigiosus* was allowed to grow over a sterile plate in the manner made use of in experiment M. A. 7. After 24 hours growth the sterile agar was given the usual point inoculation with the Sm culture and a sterile petri dish top replaced the *prodigiosus* culture.

The results of this experiment indicated that the effect of the *B. prodigiosus* was directly on the medium and the changes affected in the medium resulted in the typical Sm colony, while the control was devoid of mucoid properties.

The procedure which now suggested itself, was to look for changes in the medium exposed to the growth of *B. prodigiosus*. After repeating the first part of experiment M. A. 11 the sterile agar plate was compared with a control plate, poured at the same time and from the same batch of medium, with respect to its P_H . The P_H of the former was 7.9 while the latter (the control) was 7.1.* Apparently the product which influenced the growth was a basic substance capable of changing the P_H of the medium and thus effecting the colony structure.

Exper. M. A. 16.—A given batch of agar was dispensed to small flasks and by the addition of N/1 NaOH or N/1 HCl the P_H was adjusted to various values between 6.6 and 8.4. After sterilizing in the autoclave the P_H values were again checked; values of 6.6, 7.1, 7.3, 8.0 and 8.4 were recorded. Plates were poured, cooled and point inoculated with Sm culture. After 24 hours incubation the results were recorded.

The results were very definite in this experiment and indicated a marked favoring influence of an alkaline medium in the typical development of the Sm culture. The plates of P_H 8.0 gave a very typical colony while the plate of P_H 7.7 was essentially satisfactory but slightly more opaque than the 8.0 plate. The opacity and lack of mucoid properties increased with the decrease of P_H . The 8.4 plate was apparently too alkaline, for the resulting growth, although mucoid, was limited. Apparently a medium of a P_H of 7.8 would be best suited for the full development of the characteristics of this type of growth.

* It is to be noted that in experiments up to this time the procedure of using agar with a P_H of 7.8 did not hold. It was this work that brought about the selection of the above P_H value for mediums.

The conclusions could have been drawn and the story concluded at this point had not an experiment been performed which momentarily clouded the interpretations.

Exper. M. A. 11-b.—Two plates poured with agar of P_H 7.1 were point inoculated with Sm culture. One plate was placed in a large Novy jar and 20% CO_2 introduced. The other plate was incubated under atmospheric conditions.

The results indicated clearly that the high percentage of overhead CO_2 exerted the same influence as the alkaline medium on the production of the mucoid properties of the colony. The question that arose was: did the overhead CO_2 exert a stimulating influence on the base producing activity of the colony itself?

Exper. M. A. 19.—The above experiment was repeated with the addition of 0.002% phenol red to the agar in order to note any increase of P_H under the colony.

The results indicated a shift to an acid reaction under the colony as well as in the medium. How would it be possible to correlate these apparently contradictory tendencies of an acid substance, as CO_2 , and a basic medium in exerting a favoring influence on the development of the mucoid property of the Sm culture? If we postulate that the dissolved or combined CO_2 is responsible for stimulating the production of this very characteristic property, it is possible to understand this apparent discrepancy. For the more alkaline a medium is, the greater is its CO_2 combining power and the greater is the resulting concentration of dissolved or combined CO_2 . On the other hand, given a neutral medium, the greater the partial pressure of overhead CO_2 the greater the concentration of dissolved and combined CO_2 in the medium.

To test this experimentally, various experiments have been attempted without marked success. These experiments have had for their purpose the direct addition of carbonates to the medium. As Novy, Roehm and Soule³³ have shown, carbonates in culture mediums do not withstand autoclaving. Attempts were made to add $NaHCO_3$ directly to the partially cooled (45 C.) agar just before pouring the plates. The $NaHCO_3$ even in as small amounts as 0.1% enabled the Sm form to give an extremely mucoid colony. The controls were as usual, devoid of this property. This "proof" was only apparent for, when a drop of indicator was placed directly on the plates the intense red resulting indicated a shift of P_H from the value of 7.1, as the plates were poured, to 7.8 or 8.0. This was probably due to a decomposition of the $NaHCO_3$ which apparently takes place at incubator temperatures under the conditions of the experiment. The control plates still retained the initial

P_H . Because of the known ability of medium of 7.8 or 8.0 to foster the mucoid production by the Sm culture these experiments could not be considered as proof of the rôle of carbonates in this phenomenon.

Attempts to get around this difficulty have yielded uncertain results and leave the direct proof of the postulate, that it is a carbonate ion which is ultimately responsible for the stimulation of the mucoid production, by this form of *B. anthracis*, yet to be obtained.

Throughout this work it has been observed that all batches of mediums were not equally satisfactory, even at a P_H of 7.8, to allow a maximum development of the mucoid properties of this variant.* Attempts to attribute such differences to variations in the CO_2 combining power of the mediums have failed in experimental proof.

Recently a suggestion as to a possible explanation of this variation in mediums has presented itself. On a 2% glucose agar plate inoculated with the Sm culture, it was observed that the heavy streaks as well as the isolated colonies were absolutely devoid of the mucoid properties and were perfectly opaque. Subcultures, made from such opaque colonies, back to plain medium gave the typical Sm colony.

To follow up this lead the following experiment was performed.

Exper. M. A. 60.—Various concentrations of glucose ranging from 2% to 0.10% were added to portions of melted agar. The P_H of all mediums were adjusted to a P_H of 7.8. The control was adjusted to a P_H of 7.4† (This batch of medium maintained the Sm colony type very well even at P_H values as low as 7.4). Enough sterile litmus solution to color the agar was added to each portion of agar and plates were poured, cooled and point inoculated with Sm culture.

The results of this experiment left no doubt as to the effect of glucose and the possible manner in which it produced this effect. The colonies grown on agar containing 2%, 1% and 0.5% were devoid of all mucoid properties and were opaque. Further, beneath the colony in these cases, the litmus had turned red indicating that the production of acid, in the glucose metabolism, was a possible explanation for the effect of glucose in repressing the production of the mucoid properties of the Sm culture. In the case of the 0.10% glucose agar the colony was intermediate with respect to the control and the colonies grown on mediums containing the higher percents of glucose. An acid reaction was indicated beneath this colony also. The control colony was essentially a typical Sm colony. There was a very slight indication on the

* It has been a practice to always test a batch of medium before using it in dissociation experiments.

† The lower P_H for the control was used as a safety factor to make sure that the control had as low a P_H as any of the experimental mediums to which glucose had been added.

edge of the colony of an opaque tendency. Repetition of this experiment substituting phenol red as an indicator and using 1.00, 0.50, 0.25 and 0.12% glucose gave similar results. The colony on 0.12% glucose agar appeared to be quite devoid of the mucoid property. Similar results were obtained in the case of the Rm culture. An inspection of the accompanying photograph (fig. 4) shows the marked influence of the 0.12% glucose on the Sm culture. This was brought out by point inoculations 0.5 cm. apart on the two mediums. It is observed that

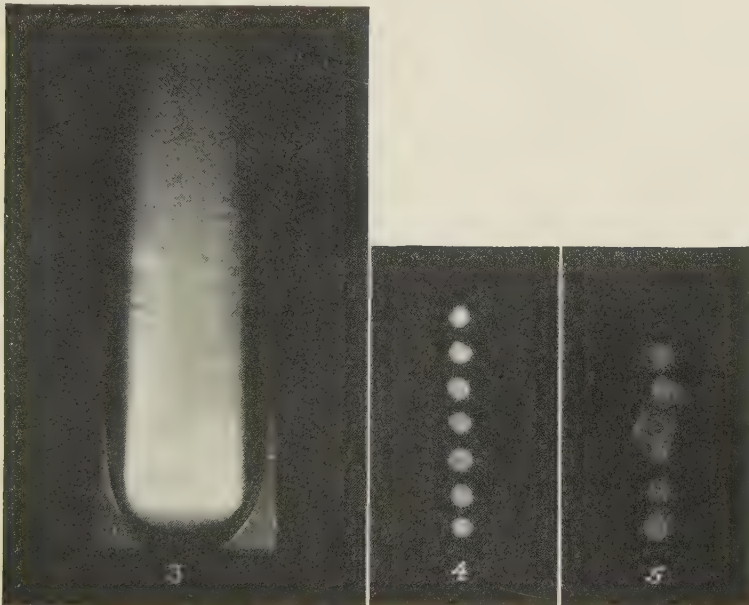


Fig. 3.—Showing nontransmissible eroded areas appearing on phantom culture in h-tubes (table 2).

Figs. 4 and 5.—Showing the inhibiting effect of the addition of 0.12% glucose to medium (fig. 4) on the production of the mucoid characteristic of the Sm form.

on the control plain agar (fig. 5), that the colonies have flowed together after 24 hours incubation while in the case of the medium containing 0.12% glucose (fig. 4) the colonies are opaque and show no tendency to flow together.

Although no data are presented here to bear out our suspicion, it is possible that variation in the effectiveness of various batches of mediums in fostering the production of the mucoid property of these forms may be linked up with variations in the amount of glucose, or

possibly glycogen, contained in the meat used for the preparation of infusion agar.

That the change in colony form of the Sm type when grown on agar with a P_H of 6.9 to 7.0 is not a permanent one is shown by the following experiment.

The Sm culture was carried along by point inoculations on plain nutrient agar with a P_H of 6.9 to 7.0 for 60 successive transplants. Transfers extending from Aug. 21, 1927, to Dec. 27, 1927, were made every 1 to 3 days. At each transfer the needle was touched to 7.8 agar, in order to note any changes of a more or less permanent nature that might have occurred as a result of serial passage on the neutral or slightly acid medium.

The results from the above experiment indicated that although a marked change of colony form was impressed on this variant by growing it on neutral or slightly acid medium for as many as 60 successive transfers, the change was not permanent. The inoculated test plates

TABLE 3.—CHANGES IN COLONY FORM OF AGAR SLANT CULTURES OF VARIANTS OF B. ANTHRACIS SEALED FOR 14 MONTHS

Variant Inoculated	R		RS		S		Rm		Sm		Rp		Sp	
Colony form	R	%	R	%	S	%	Rm	%	Sm	%	R	%	RS	%
Recovered		100	RS	30	R	20	R	20	S	90		100		100
			S	40	R	80		80		10				
				30										

(agar P_H 7.8) always gave the typical mucoid colony throughout the experiment.

Although there are many interesting and suggestive aspects involved in the production or failure to produce the markedly mucoid and transparent type of growth, as related to certain defined chemical conditions of the medium, the only use made of these findings at this time is to aid in standardizing the medium used so as to insure the manifestation of this characteristic. Hence since January, 1927, all stock cultures of the anthrax variants have been carried on agar plates with a P_H of 7.8.

PRESERVATION OF VARIANTS IN SEALED CULTURES

In connection with maintaining the stock of these variants, reference should be called to the following experiment in which the variants were kept on agar slants, sealed with wax, at icebox temperature for 14 months.

Exper. A 41.—On Oct. 20, 1926, the variants were inoculated on plain agar slants. After 24 hours growth of 37 C. the slants were sealed with sealing wax and placed in the cold room (15 C.).

These were opened on Dec. 7, 1927, and subcultures made to agar plates, the results are given in table 3.

In maintaining these variant cultures of *B. anthracis* the method of certainty is the plate method with frequent transfers.

DISSOCIATION EXPERIMENTS

The following study of the change from one colony form to another had two purposes. First to determine a sequence of these various colony types in passing from one colony form to another. Many of the recent workers have focused their attention on the R and S types, and colony forms dissimilar to these have often been described as "intermediate forms." There has also developed (Hadley³) a line of thought tending to treat bacterial variants as stages in a life cycle of the organism rather than as variants or mutations. Apparently with the seven variants at hand an excellent opportunity presented itself to find evidence of a cycle, if such was involved, in the change from one colony type to another.

A second object of this phase of the work was to determine culture conditions in which dissociation of the various forms would occur. To present all of the data accumulated on this phase of the work would require too much space. These tests involve a study of over 250 cultures of the different forms under various experimental conditions. From these 250 cultures over 3500 plates have been streaked in order to determine the colony type present at intervals during the experiments. The selection of the data to be presented is made so as to bring out the effect of different culture conditions on dissociation and also tables are selected which show how the various forms react under dissociating conditions. Finally summarizing tables, resulting from a study of all of the available dissociation data, are presented which make possible conclusions on the first object of these experiments; namely, the trend of dissociation in *B. anthracis*.

Liquid and Solid Mediums.—The first observed modifications of *B. anthracis*, which involved mainly changes in virulence and morphology, were effected in liquid mediums, (Toussaint,³⁴ Pasteur,⁴ Chamberland and Roux,⁸ etc.). Preisz⁵ used broth in producing experimental modifications characterized by changes in colony formation as well as changes in morphology and virulence. Of more direct application to the general problem of microbic dissociation are the recent results of Arkwright,¹ DeKruif,² and Soule,²² who have found liquid mediums superior to solid mediums for producing the R form from the S type

34. Compt. rend. Acad. d. Sc., 1880, 91, p. 303.

of culture of the colon-typhoid organisms, *B. leptisepticum* and *B. subtilis*, respectively.

Exper. A 1.—This experiment simply involved the inoculation of broth and agar slants with the variants. These cultures were placed in the hot-room for 24

TABLE 4.—THE EFFECT OF SOLID AND LIQUID MEDIUMS ON THE DISSOCIATION OF THE VARIOUS FORMS OF *B. ANTHRACIS*
(44 Days' Incubation)

Form	Agar Slant Wax Seal		Broth	
		%		%
R.....	R	100	R	100
RS.....	RS	100	RS	60
			R	20
			S	10
			Sm	10
S.....	S	70	S	30
	RS	30	R	70
Rm.....	R Rm	100	Rm	50
			R-Rm	50
Sm.....	S	100	Sm	5
			S	80
			RS	15
Rp.....	Rp	100	Rp	100
Sp.....	Sp	95	Sp	70
	Sp-S	5	Rp	30

TABLE 5.—THE EFFECT OF VOLUME ON THE DISSOCIATION OF THE R AND S FORMS IN 10% NORMAL RABBIT SERUM

Days	R				S			
	10 cc.		300 cc.		10 cc.		300 cc.	
	R	%	R	%	S	%	S	%
0.....	R	100	R	100	S	100	S	100
4.....	R	100	R	100	S	98	S	50
					Sm	2	R	50
7.....	R	100	R	50	S	90	S	50
			RS	50	R	10	R	50
13.....	R	90	R	75	S	90	S-Sm	50
	RS	10	Sm	25	R	10	Rm	50
14.....	R	50	R	66	S	5	S	25
	RS	50	Sm	30	R	95	Sm	25
			S	4			R	50
16.....	R	50	R	50	S	85	S	40
	RS	50	Sm	40	Sm	5	Sm	10
			S	10	R	10	R	50

hours. The agar cultures were then sealed with wax to prevent drying and replaced in the hot room. Forty-four days later the agar slant and broth cultures were plated and the resulting types of colonies recorded in table 4.

The results agree in general with those of earlier workers. When the ease with which these variants have been carried for 2 years on solid medium is considered, the advantage of solid medium for maintaining a variant becomes even more convincing.

Volume.—Soule²² has recently made use of relatively large volumes of liquid medium to sponsor dissociation. He bases the idea on the fact that a large volume increases the duration of active multiplication of the organisms and hence the chances for dissociation.

Exper. A 11.—620 cc. of 10% normal rabbit serum in broth was dispensed in two 10 cc. amounts in tubes and two 300 cc. amounts in sterile Florence flasks. After incubation to insure sterility, a tube and flask were inoculated with the R form. The other tube and flask were inoculated with the S form.

Table 5 gives the results as indicated by plates streaked at intervals from these cultures. This table, besides confirming in general Soule's findings, brings out certain facts characteristic of the dissociation phenomena in *B. anthracis* as reported in this work. In the first place the seven variants as described do not represent an exclusive list of possible variations in colony forms observed, but they do represent certain limits with respect to certain colony characteristics toward which the anthrax culture studied may tend to finally attain when subjected to dissociating influences. There have been numerous observations of colony forms with colony properties intermediate between certain variants. It is often noted that such colony forms appear in a culture just prior to the occurrence of the form for which the intermediate appears to be the precursor. Such an example occurs in the 300 cc. S culture in table 5. It is noted that on the thirteenth day plates from the culture showed a colony form with characteristics between the S and Sm. Plates from this culture on the fourteenth day showed the characteristic Sm colony form.

An examination of other tables as they are presented, will show that the presence of such intermediate forms frequently herald the appearance of a new form, whose colony properties and those of the original culture are shared by the intermediate.

The question of the sudden or gradual change of organisms from one type to another has received attention favoring both views. Arkwright¹ emphasized the occurrence of intermediate forms when he states "The variety of colonies met with is so great and the difference between them often so indefinite, that attention has purposely been directed only to the more obvious characteristics which are definitely associated with especial behavior in broth cultures, and emulsion in salt solution." DeKruif² on the other hand has emphasized the sudden change from one form to another. Hadley³ has in his earlier work done likewise,

but in more recent work³⁵ has observed the gradual change from one form to another.

A second item to be noted in this table, which is also to be observed in other tables, is the irregularity of the percentage occurrence of a given variant from one plating to the next. For instance, in the 10 cc. S culture the last platings show the R form present in the culture in 10%, 95% and 10% amounts. Although such precautions as shaking well before streaking were always observed, this type of "error" has persisted throughout the work. It may be possible that such "errors" represent the actual state of affairs with respect to the organisms themselves since such results are met with primarily in actively dissociating cultures.

A fourth item of interest brought out in this work is the ability to pass from R to S, as well as from S to R. The direction of the vast majority of reported variation has been from the S to R. However, Preisz⁵ in obtaining his homogeneous colony from the usual rough anthrax form by cultivation at 42.5 C. effected the reversion of R to S. Jordan³⁶ has effected a change from the experimentally derived R form of *paratyphosus bacillus* back to the S form. Soule²² has done the same with *B. subtilis*. Although there seems to be no rigid barriers as to the direction in which variations may occur, in the case of *B. anthracis*, it will be noted that certain forms are more stable than others. The R form as noted in this table, is more stable than the S form. This will be seen even better in later tables.

Glucose Broth.—A study of the dissociation of the variants of *B. anthracis* in liquid medium of varying composition will be next considered. The effect of the addition of glucose to plain broth appeared to be of some interest, because of the recognized differences in rate of growth as well as keeping qualities of cultures in broth with and without glucose present. The question was thus raised as to the effect of the addition of glucose to medium on the dissociation of the variants of *B. anthracis*.

Exper. A 16.—A given lot of broth was divided into two batches. To one batch 2 gm. of glucose per 100 cc. of broth was added. After dispensing the two batches into tubes (10 cc. each) and sterilizing, they were incubated to insure sterility. Each of the variants were then inoculated into a tube of plain broth and one of glucose broth which were placed in the incubator. At stated intervals plates were streaked to determine the variants present.

35. Personal communication.

36. Proc. Soc. Exper. Biol. & Med., 1926, 23, p. 762.

Table 6, giving the results on three of the most stable variants, illustrates the greater dissociating value of glucose broth over plain broth. It is to be noted that the Sp form gave rise to an S variant while the Rp form gave an R colony form after a month's incubation. This fact will later become significant in attempting to determine the trend of dissociation.

Experimental data, to be presented on the rôle of other factors affecting the dissociation of the anthrax bacillus, were obtained in a comprehensive dissociation experiment which served to repeat many earlier experiments. These results become especially significant when it is noted that the stock of variants from which the inoculum for this

TABLE 6.—THE EFFECT OF AGING THE R, Rp AND Sp FORMS IN BROTH AND 2% GLUCOSE BROTH

Days	R				Rp				Sp			
	Plain		Glucose		Plain		Glucose		Plain		Glucose	
		%		%		%		%		%		%
0.....	R	100	R	100	Rp	100	Rp	100	Sp	100	Sp	100
1.....	R	100	R	90	Rp	100	Rp	60	Sp	100	Sp	100
			S	10			Sp	40				
2.....	R	100	R	50	Rp	100	Rp	80	Sp	100	Sp	100
			S	50			Sp	20				
3.....	R	100	R	30	Rp	100	Rp	50	Sp	100	Sp	100
			RS	70			Sp	50				
4.....	R	100	R	30	Rp	100	No growth		Sp-Rp	100	Sp-Rp	100
			RS	70								
6.....	R	100	R	60	Rp	100	Rp	100	Sp	100	Sp	100
			RS	40								
11.....	R	100	R	70	Rp	100	Rp	100	Sp	100	Sp	98
			RS	30							Rp	2
31.....	R	100	R	100	Rp	90	No growth		Sp	100	Sp	10
					R	10					S	90

test was obtained, had been constantly subcultured every 1 to 3 days on agar plates for a period of a year and a half. A large portion of such transfers were from isolated colonies typical of the form.

For the purpose of brevity the general details of this experiment will be followed by a presentation of typical portions of the results. The discussion of the results of the different parts of the experiment will then be taken up under the headings of the various factors affecting dissociation.

Exper. A 24.—One lot of broth was used for all cultures in this test and the recorded P_H values are those determined just before the inoculation. In the case of the milk medium (tables 10 and 11) fresh milk was neutralized by the addition of N/1 NaOH and autoclaved at 110 C. for 20 minutes. After standing in the cold room over night the lower layer of milk was siphoned off, tubed in 10 cc. portions and sterilized.

After the inoculation of the various culture forms into the different mediums, most of the cultures were kept in the hot room. To determine the effect of temperature on the dissociation reaction, four sets of cultures of the variants in broth were placed at temperatures other than 37, 5, 12, 26, and 42.5 C. All of these temperature conditions, except the 26 C., were thermostatically controlled. The 26 C. temperature was obtained in an inner room and the temperature remained very constant as shown by a recording thermometer placed beside the cultures. Frequent observation indicated a satisfactory small limit of variation (1 to 2 degrees) in the case of the 5 and 12 cold rooms. The 42.5 C. incubator held within a half degree and frequently less until after the third week of the experiment when it failed in operation. This phase of the experiment was then discontinued.

The essential technic of this experiment was similar to former aging experiments in that the stated culture form was incubated under conditions defined in the tables and the results were determined by plating from the culture at recorded intervals. An additional feature of this experiment was the transplanting of the dissociating culture into fresh medium at the end of 18 days. Platings were made from the new culture as well as the old during the remainder of the test.

Reaction.—DeKruif² claims a favoring influence of an alkaline reaction (8.5) over an acid reaction (6.0) in producing variation of *B. leptisepticum*. Hadley³⁷ likewise reports the favoring influence of a medium with a pH of 7.8 on the dissociation of *B. pyocyaneus*. On the other hand Eisenberg¹⁴ has made use of glycerol agar to produce asporogenic races of *B. anthracis*. He attributed the change to acid formed as the result of the utilization of the glycerol for food. Soule²² has definitely shown the favoring influence of an acid over an alkaline reaction in producing dissociation in the case of *B. subtilis*.

Because of the favoring influence of an acid forming substance as glucose, on the dissociation of *B. anthracis* it was of interest to determine whether there was a difference in the dissociating effect of alkaline, neutral or acid mediums. Tables 7, 8, 10 and 11 indicated the possibility of dissociation occurring in alkaline, neutral or acid broth with the acid broth showing somewhat the greater dissociating influence. To judge quantitatively what effect a set of conditions may have in influencing dissociation in the case of *B. anthracis*, is very difficult. The percentage change in a culture is not sufficient, for one change may not be as deeply seated as another. For example, a change of R to RS is accomplished more readily than a change of R to S. Hence in evaluating factors influencing dissociation consideration must be given to the qualitative, as well as quantitative aspects of the change.

37. J. Infect. Dis., 1924, 34, p. 260.

TABLE 7.—THE EFFECT OF AGING THE Rp FORM IN BROTH OF VARIOUS PH VALUES;
ALSO THE EFFECT OF TRANSPLANTING ON A DISSOCIATING CULTURE

Days	Broth 6.3				Broth 7.4				Broth 8.2			
	Rp	%		%	Rp	%		%	Rp	%		%
0.....	Rp	100			Rp	100			Rp	100		
2.....	Rp	100			Rp	100			Rp	100		
4.....	Rp	100			Rp	100			Rp	100		
9.....	Rp	100			Rp	100			Rp	100		
12.....	Rp S*	50 50			Rp	100			Rp Sp	90 10		
15.....	Rp S*	50 50			Rp Sm	99 1			Rp	100		
18.....	Rp S*	50 50		†	Rp	100		†	Rp	100		†
22.....	Rp S*	90 10	Rp S*	80 20	Rp	100	Rp	100	Rp	100	Rp	100
25.....	Rp	100	Rp S*	50 50	Rp	100	Rp	100	Rp Sp	50 50	Rp	100
32.....	Rp	100	Rp R	30 70	Rp	100	Rp	100	Rp	100	Rp	100
44.....	Rp S*	95 5	R	100	Rp	100	Rp	100	Rp	100	Rp	100

* S colonies typical except for size. These colonies ranged from .2 to .5 mm.

† One loop transfer on 18th day from original tube to fresh tube of the same medium.

TABLE 8.—THE EFFECT OF AGING THE Sp FORM IN BROTH OF VARIOUS PH VALUES;
ALSO THE EFFECT OF TRANSPLANTING ON A DISSOCIATING CULTURE

Days	Broth 6.3				Broth 7.4				Broth 8.2			
	Sp	%		%	Sp	%		%	Sp	%		%
0.....	Sp	100			Sp	100			Sp	100		
2.....	Sp	100			Sp	100			Sp	100		
4.....	Sp	100			Sp	100			Sp	100		
9.....	Sp	100			Sp Sp-Rp	60 40			Sp-Rp	100		
12.....	Sp-Rp	100			Sp Sp-Rp	80 20			Sp Rp	80 20		
15.....	Sp	100			Sp S-Sp	80 20			Sp Rp	80 20		
18.....	Sp-Rp	100		†	Sp	100		†	Sp Rp	70 30		†
22.....	Sp	100	Sp S*	90 10	Sp	100	Sp Sp-S	98 2	Sp	100	Sp RS	70 30
25.....	Sp	100	Sp	100	Sp	100	Sp	100	Sp Sp-Rp Rp	40 30 30	Sp	100
32.....	Sp	100	Sp	100	Sp	100	Sp Sp-Rp	20 80	Sp Sp-Rp	70 30	Sp Rp	70 30
44.....	Sp S	90 10	R	100	Sp Rp	70 30	R	100	Rp†	100	Sp Rp Sp-Rp	30 30 40

* S colonies typical except for size. These colonies ranged from 0.2 to 0.5 mm.

† Very small colonies.

† One loopful transfer on 18th day from original tube to fresh tube of the same medium.

TABLE 9.—THE EFFECT OF BROTH, 0.1% PHENOL BROTH AND 10% NORMAL RABBIT SERUM
BROTH ON THE DISSOCIATION OF THE Rm FORM; ALSO THE EFFECT OF TRANS-
PLANTING ON A DISSOCIATING CULTURE

Days	Broth 7.4				0.1% Phenol Broth							
		%		%		%		%		%		%
0.....	Rm	100			Rm	100			Rm	100		
2.....	Rm	100			Rm	100			Rm	100		
4.....	Rm	100			Rm	50			Rm	95		
					S	50			Rm-R	5		
9.....	Rm	80			Rm	40			Rm	93		
	R	20			Rm-Sm	50			Sm	2		
					Sm	10			R	5		
12.....	Rm	80			Sm	30			Rm	80		
	R	20			RS	10			R	20		
					S	60						
15.....	Rm-R	100			R	5			Rm-R	100		
					S	95						
18.....	Rm	10			S	50			Rm	70		
	Rm-R	60			Sp	50			Rm-R	30		
	R	30										
22.....	Rm	5	Rm	5	S	30	S	100	Rm	80	Rm	40
	Rm-R	95	R	95	RS	70			Rm-R	20	Rm-R	60
25.....	Rm	5	R	100	S	100	S	100	Rm	100	Rm	100
	Rm-R	80										
	R-Rp	15										
32.....	R	100	R	50	S	100	S	70	Rm-R	100	Rm-R	40
			Rm-R	50			RS	30			R	60
44.....	Rm	50	Rm	30	S	100	S	50	Rm-R	50	Rm-R	50
	Rm-R	50	R	70			RS	50	Rm-R	50	Rm-Sm	50

TABLE 10.—THE EFFECT OF BROTH OF VARYING PH, 2% GLUCOSE BROTH, 0.1% PHENOL
BROTH, MILK AND 10% NORMAL RABBIT SERUM BROTH ON THE DISSOCIATION
OF THE R FORM

Days	Broth 8.2		Broth 7.4		Broth 6.3		2% Glucose Broth 7.1		0.1% Phenol Broth 7.3		Milk 7.3		10% Serum Broth	
		%		%		%		%		%		%		%
0.....	R	100	R	100	R	100	R	100	R	100	R	100	R	100
2.....	R	100	R	100	R	100	R	95	R	100	R	100	R	100
							RS	5						
4.....	R	100	R	98	R	100	R	50	R	40	R	99	R	100
			RS	2			S	50	RS	60	Rm	1		
9.....	R	100	R	100	R	100	R	25	R	30	R	98	R	100
							S	75	RS	50	Rp	2		
									S	20				
12.....	R	100	R	100	R	98	R	10	R	5	R	100	R	100
					RS	1	RS	5	S	95				
					S	1	S	85						
15.....	R	50	R	100	R	99	R	10	R	30	R	70	R	100
	RS	50			S	1	S	90	RS	30	Rm-Sm	30		
									RS	20				
									S	20				
18.....	R	100	R	100	R	100	RS	5	R	20	R	100	R	100
							S	90	RS	10				
							Sp	5	S	70				
22.....	R	100	R	100	R	95	R	5	RS	20	R	30	R	89
					Rm	5	RS	20	S	80	RS	58	Rm	1
							RS	60			S	2	R-Rp	10
							S	15			S-Sm	10		
25.....	R	100	R	100	R	100	S	100	R	25	R	80	R	80
									RS	25	Rm	20	R-Rm	20
									S	50				
32.....	R	100	R	100	R	100	S	100	RS	30	R	90	R	100
									S	70	S	10		
44.....	R	90	R	100	R	100	R	100	S	100	R	95	R	95
	RS	10									S	5	S	5

Transplanting the dissociating cultures after 18 days and testing the effect of aging on the new culture as well as the old, showed in some cases an increase of dissociation, in others no increase and in but few cases were there parallel tendencies of direction of dissociation.

TABLE 11.—THE EFFECT OF BROTH OF VARYING PH, 2% GLUCOSE BROTH, 0.1% PHENOL BROTH, MILK AND 10% NORMAL RABBIT SERUM BROTH ON THE DISSOCIATION OF THE S FORM

Days	Broth 8.2		Broth 7.4		Broth 6.3		2% Glucose Broth 7.1		0.1% Phenol Broth 7.3		Milk 7.3		10% Serum Broth	
	S	%	S	%	S	%	S	%	S	%	S	%	S	%
0.....	S	100	S	100	S	100	S	100	S	90	S	80	S	80
	RS	10	RS	20			RS	10	RS	10	RS	20	RS	20
2.....	S	50	S	80	S	100	S	100	S	100	S	100	S	100
	RS	50	RS	20										
4.....	S	70	RS	100	S	100	S	85	S	90	S	80	S	100
	RS	30					RS	10	RS	10	RS	20		
							RS	5						
9.....	S	65	S	60	S	30	S	70	S	100	S	70	S	88
	RS	30	RS	40	RS	60	RS	20			RS	10	RS	10
	R	5			R	10	R	10			R	20	R	2
12.....	S	50	S	100	S	100	S	80	S	100	S	50	S	50
	R-Rp	50					RS	20			RS	20	RS	10
											R	30	R	40
15.....	S	50	S	90	S	100	S	90	S	100	S	85	S	30
	RS	25	R	10			RS	10			R	15	RS	30
	R-Rp	25										R	40	
18.....	S	20	S	20	R	100	S	5	S	80	S	90	S	1
	RS	30	R	80			RS	20	RS	20	R	10	RS	99
	R-Rp	50					RS	60						
							RS	15						
22.....	RS	1	RS	10	RS	15	RS	100	S	10	S	60	RS	10
	R-Rp	99	R	90	RS	40			RS	80	RS	40	R	90
					RS	25			RS	10				
					R	20								
25.....	R*	100	S	5	R-Rm	100	S	20	S	100	S	100	S	50
			RS	95			RS	80					RS	50
32.....	R*	100	S	60	R	100	No growth		S	80	S	40	R	100
			R*	40					RS	20	RS	30		
											Sp	20		
											Rp	10		
44.....	S†	60	S	30	R	100	1 colony		S	5	S	45	R	100
	RS†	40	R*	70			RS		RS	95	RS	45		
											Sp	10		

* By transmitted light these colonies appeared as typical R forms; by reflected light, however, the surface was slightly moist and not the usual dull gray or a true R.

† Colonies smaller than usual; about 0.5 mm. in diameter.

Phenol.—Growing the anthrax variants in 0.1% phenol broth has proved to be one of the best methods of producing changes in this organism. Tables 9-11 show this effect. A very interesting feature of the phenol dissociation is the marked tendency which exists in “directing” the dissociation toward the S form. This was noted in the case of the RS and Rm as well as the R form.

Milk.—In the course of tests to ascertain the type of variant present in milk cultures, it was noted, with some surprise, that considerable dissociation at times occurred. This early observation has been verified as will be seen from tables 10-11 which show the influence of prolonged incubation on the dissociation of milk cultures of the R and S forms.

It is to be noted that milk is an excellent medium for producing dissociation in the case of *B. anthracis*.

Temperature.—The rôle of temperature in producing variants has been used since the days of Pasteur. In later years Preisz⁵ (1911) made use of incubation at 42.5 C. to produce variations from a normal virulent culture. Soule²² has shown that no dissociation occurred at 5 C. and very little at temperatures less than 25 C. in the case of *B. subtilis*. He obtained his maximum dissociation at 45 C.

The results of this investigation indicate in general, a marked decrease in dissociation activity at the reduced temperatures of 5 and 12 C. Dissociation occurred at 26 and 37 C. while at 42 C. it may be said in general that it was greatest. The results indicating variations in colony form were, however, somewhat disappointing in the case of the R form. It had been anticipated that mucoid forms would be readily found since Preisz¹¹ had obtained mucoid colony types in Pasteur vaccines. Later, Preisz⁵ obtained similar forms by growing a normal virulent anthrax culture in broth, to which no peptone or salt had been added, at 42.5 C. When in this work the factors of peptone-free broth and higher temperature of incubation were combined the Rm type of growth was obtained from the R type. The controls (plain broth cultures grown at 37 C. and 42 C. as well as a peptone free broth culture grown at 37 C.) showed only limited dissociation.

Serums.—Considerable work has appeared in recent years dealing with the role of immune serums in producing variations in bacteria (Stryker,³⁸ Griffith,³⁹ Jacobson and Falk,⁴⁰ Soule,²² etc.). It was decided to determine the effect of serums of laboratory animals with varying natural resistance to anthrax as well as "immune" rabbit serum on the dissociation of the variants of *B. anthracis*. The results on the first aspect of this problem will be reported in this paper.

Exper. A21.—Normal serum was obtained from white rats (more or less resistant to anthrax), guinea-pigs (readily susceptible to anthrax), and rabbits (somewhat resistant to anthrax).

38. J. Exper. Med., 1916, 24, p. 49.

39. Report 18 on Public Health and Medical Subjects, Ministry of Health, 1923.

40. Proc. Soc. Exper. Biol. & Med., 1926, 23, p. 785.

Two normal rabbits were bled from the carotid according to the technic described by Novy.⁴¹ The blood was allowed to clot and the serum collected and pooled.

Guinea-pig and white rat serum was obtained in an aseptic manner from blood drawn from the exposed heart as described by Novy.⁴¹ The serum of 4 guinea-pigs was pooled and the serum of 12 white rats was pooled before use. All serum was collected after allowing the blood to clot.

The various serums were diluted in broth 1:10 and dispensed aseptically to small sterile test tubes. The precaution of incubating all mediums before use was always observed.

The initial plan for this experiment was to make daily transfers of each of the variants in plain broth, normal rabbit serum broth, guinea-pig serum broth and white rat serum broth. This was modified because it was found that the normal serum of the white rat and rabbit exerted such a marked inhibiting influence on these cultures that it was difficult to get an appreciable growth in less than 3 to 4 days. Hence transfers were made at 3 to 5 day intervals. Ten serial transfers were made for each variant in each medium. Plates were streaked at the time transfers were made in order to note the changes effected in the cultures.

The results of this experiment obtained on the R form are to be found in table 12. Serums in general appeared to foster dissociation. Rat serum seemed especially beneficial in this respect and guinea-pig serum seemed least.

Daily Transfers. Continued Incubation and Growth in Filtrates of Old Cultures.—From the work so far it appears that dissociation is usually most marked when conditions are adverse to the optimum growth of the organism. This fact has been suggested in the majority of studies dealing with microbic dissociation. In order to substantiate this fact the following experiment was performed.

Exper. A 28.—A 300 cc. flask of a 70-day old broth culture of the RS form was filtered through a Berkefeld N filter. After incubation to test for sterility this was aseptically dispensed in 5 cc. amounts to sterile tubes. Broth was dispensed in similar amounts to tubes and sterilized. (The P_H of both was 7.6).

The test was set up in three parts. The first part consisted in daily transfers of the variants in broth. The second and third part consisted of continued incubation of the variants in broth and filtrate of the RS culture, respectively. Plates were streaked from these cultures at stated intervals.

Table 13 shows the results in the case of the RS variant. These results indicate that continued incubation results in greater dissociation than daily transfers in the case of *B. anthracis*. Although the filtrate of RS appeared to be somewhat more active than plain broth in producing dissociation, the difference was not as great as might have been expected. An interesting fact brought out in this experiment was the ability of a filtrate from a 70-day old culture to support growth, which was, however, markedly slower than that of the control in broth.

41. Laboratory work in Bacteriology, 1899, Wahr, Ann Arbor, p. 461, J. Infect. Dis., 1917, 20, p. 502.

TABLE 12.—THE EFFECT OF SERIAL TRANSFER IN BROTH, NORMAL RABBIT, GUINEA-PIG AND RAT SERUM BROTH ON THE DISSOCIATION OF THE R FORM

Transfer	Broth		Normal Rabbit Serum Broth		Normal Guinea-Pig Serum Broth		Normal Rat Serum Broth	
	R	% 100	R	% 100	R	% 100	R	% 100
0.....	R	100	R	100	R	100	R	100
1.....	R	100	R	100	R Rm	90 10	R Rm	85 15
2.....	R	100	R	100	R	100	R Rm	70 30
3.....	R	100	R Rp	90 10	R	100	R Rm	60 40
4.....	R	100	R Rp	95 5	R	100	R Rm-Sm	70 30
5.....	R	100	R	100	R RS	10 90	R Rm-Sm Rm Sm	30 30 10 30
6.....	R	100	R	100	R RS	85 15	R Sm	60 40
7.....	R RS	50 50	RS R-Rp	5 95	R	100	R Rm Rm-Sm	5 55 40
8.....	R RS	50 50	R RS Rm	10 50 40	R RS S	70 20 10	R R-Rm Rm Rm-Sm	5 30 30 35
9.....	R RS	50 50	R RS	20 80	R RS	80 20	R R-Rm Rm-Sm	5 55 40
10.....	R	100	Rm-Sm S-Sm	50 50	R	100	R R-Rm Rm Rm-Sm	10 50 10 30

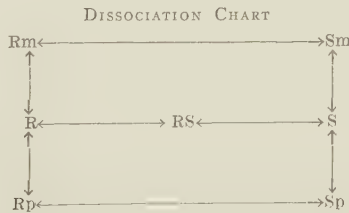
TABLE 13.—THE EFFECT OF AGING THE RS FORM IN BROTH AND FILTRATE OF A 70-DAY RS BROTH CULTURE; ALSO DAILY TRANSFER OF THIS FORM IN BROTH

Days	0		2		8		16	
	RS	% 100	RS	% 100	RS	% 100	RS	% 100
Daily transfer in broth.....	RS	100	RS	100	RS	100	RS	100
Aging in broth.....	RS	100	RS RS	90 10	RS	100	RS RS R	20 20 60
Aging in RS filtrate....	RS	100	RS RS RS	20 10 70	RS S	5 95	RS RS S R	30 30 10 30

TREND OF DISSOCIATION

It might appear that this paper has piled confusion onto an already confused state of affairs in regard to bacterial variation. So it has seemed to the author throughout the active period of investigation. The results were recorded and although they appeared interesting and varied, they pointed not to clarification but to confusion. With the completion of the major portion of the work just reported, on the effect of various factors influencing dissociation, a careful study was made of the results. In this, the emphasis was not placed on the manner of producing dissociation, for obviously this occurred under a wide range of conditions, but rather on the trend of dissociation starting with various forms.

From this study certain marked tendencies became apparent. First, the changes from one colony form to another were as a rule not sudden but were gradual with the occurrence of many intermediate forms. Secondly, it appeared that all dissociation tendencies were reversible and in the reversion the same intermediate colonies appeared as in the first reaction. In the third place it seemed that the R and S colony types represented one possible direction of dissociation. Further, that this simple scheme could be elaborated to cover additional facts of variation by simply associating with the rough or smooth characteristics such properties as a mucoid or phantom growth characteristic. In these cases an R form may acquire the mucoid type of growth and become an Rm form. This may revert to the R form or may lose its rough properties, retaining the mucoid property, and become an Sm form. This in turn may revert to the Sm culture type or retain its smooth properties and lose its mucoid properties and become an S culture type. An analogous series of changes can occur involving the phantom forms. The following schematic diagram may clarify the above interpretation of the trend of observed dissociation of the variants of *B. anthracis*.



To test this interpretation, a scheme of analyzing the dissociation results, some of which have been presented in the foregoing tables, was

devised and applied to a large portion of the dissociation results at hand. The scheme for this test involved the construction of seven tables, (one for each variant). Reference to table 14 will aid in the explanation. This table was constructed after examining the records of 39 cultures of the R form. These cultures were maintained under various conditions which resulted in the dissociation of the R form into other forms. From 24 of the cultures, the RS form was isolated at one time or another. From 14 of the 39 R cultures the S form was isolated. From 8 of the 39 R cultures the Rm form was isolated, etc. Further, in the 24 cases in which the RS form was isolated from a culture, originally R, other forms accompanied* the RS form. In 15 of the 24 cultures only the R type was isolated with the RS form.

TABLE 14.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN R CULTURES (39).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	RS (24)	S (14)	RM (8)	Sm (4)	Rp (7)	Sp (3)
	R (15)	R, RS (6)	R (4)	R, RS (1)	R (5)	RS, S (1)
	R, S (5)	R (5)	R, RS (1)	R, RS, Rm-Sm (1)	R, RS (1)	R, Rp S (1)
	R, Rm (2)	R, Sm (1)	R, Sm (1)	R, Rm, Rm-Sm (1)	R, RS, S, Sp (1)	R, RS, S Rp (1)
	R, R-Rp (1)	R, RS, S-Sm (1)	R, <u>R</u> -Rp (1)	R, Rm-Sm (1)		
	R, S-Sm (1)	R, RS, Rm-Sm (1)	R, RS, R-Rp (1)			
		R, Rp, Sp (1)				

The figure in () indicates the number of times a given variant or combination of variants was found.

R cultures which developed no variants are not given in the table.

In 5 cases the R and S forms accompanied the RS form. In 2 cases the R and Rm forms accompanied the RS form, etc.

The significance to be attached to the accompanying forms is that they may represent forms from which the variant in question originated. It must be remembered that such precursors may not appear in all experimental cases for the obvious reason that they might not have been present in the dissociating culture at the particular time the cultures were streaked onto plates.

It is now possible to analyze the results and test the interpretation as schematically represented in the dissociation chart.

Example 1.—From the chart we would expect to obtain directly from the R culture the forms Rm, Rp and S and the forms Sm and Sp only after other forms as S or Rm or Rp had occurred as possible precursors. By examination of table 14

* Accompanying forms are those occurring on the same, or immediately preceding plate on which the variant in question (at the head of the column) was isolated from the culture.

it is observed at a glance that the RS form, an intermediate between the R and S types, occurs most frequently. Next in frequency of occurrence comes the S, then the Rm and Rp. It is noted that the occurrence of these forms have frequently no other precursors than the R form. Of less frequent occurrence are the Sm and Sp forms. An examination of the accompanying forms present when these forms were isolated is interesting. In 3 of the 4 isolations of the Sm form from cultures originally R, the Rm-Sm type of colony was simultaneously isolated. This suggests that the original R form might have changed first to the Rm and then to the Sm form as indicated in the dissociation chart. In all of the three occurrences of the Sp type the S form accompanied its isolation. In two of these cases the Rp form was also isolated, indicating two possible courses for the dissociation of the R to the Sp form as suggested by the chart.

Example 2.—A second example will be analyzed selecting a culture form on one of the "corners" of the diagram, namely, the Sm form. Because of the suspected position of this culture with respect to the other forms as represented by this diagram it is anticipated that such forms as Rm and S may occur directly from the Sm form, while the occurrence of R, RS, Rp and Sp forms should require the intermediate appearance of other forms as precursors. It is observed from table 18 that although the R form appears ultimately in 13 different cultures of Sm, in every case there is an experimentally observed possible precursor (the Rm or S form). While in the longer list of occurrences of the S form the majority offer only the possibility of the Sm as being the precursor. The same holds for the occurrence of the Rm. The single appearance of an Rp form occurred with the possible indicated routes of approach being Sm to S to Sp to Rp, or Sm to S to RS to R to Rp. The Sp occurred with an indicated route Sm to S to Sp.

Similarly, tables 15-20 indicate a likelihood that the dissociation chart represents a scheme not far from the truth as related to the trend of dissociation in *B. anthracis*. When the possibility of experimentally missing the occurrence of the precursors, as already mentioned, is considered, the correlation between observed facts and the schematic representation are even more striking.

A point to be emphasized is that the indicated scheme apparently deals with sharply defined cultural entities. This of course is not true. All forms represent characteristics not of point definiteness but rather of broader, but nevertheless real limits. Further the actual schematic representation of passage from one form to another may not be along a simple rectangular course, but along a more or less diagonal one, of which the hypothetical rectangular course is but the projection of the true one. But for the present such complicating, although without a doubt more accurate considerations can be left for the future when further material warrants a more exact consideration.

A further tendency is noted in the dissociation of *B. anthracis*. More or less frequently a dissociating culture tends ultimately to the R form. One reason for this may be that the R form is the most stable* and is

* The phantoms are also quite stable.

TABLE 15.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN RS CULTURES (24).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (23)	S (19)	Rm (5)	Sm (6)	Rp (3)	Sp (0)
	RS (15)	RS (10)	RS, R (4)	RS, S (2)	R, RS (1)	
	RS, S (8)	RS, R (8)	Rm-Sm (1)	S (1)	RS, S (1)	
		RS, R, Sm (1)		RS, R-Rm, R-Rp (1)	R, RS, S (1)	
				RS, S, R (1)		
				R-Rm (1)		

The figure in () indicates the number of times a given variant or combination of variants was found. The RS culture which developed no variants is not given in the table.

TABLE 16.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN S CULTURES (39).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (36)	RS (36)	Rm (3)	Sm (5)	Rp (3)	Sp (3)
	S (13)	S (21)	S, RS (1)	S (3)	R (1)	S, RS, (1)
	S, RS (19)	S, R (14)	S, RS, R (1)	S, R (1)	RS, RS, S, R-Rp (1)	S, RS, R (1)
	S, Sm (3)	S, Sp (1)	S-Sm, S, R (1)	S-Sm, R, Rm (1)	S, RS, Sp (1)	S, RS, Rp (1)
	RS, R-Rp (1)					

The figure in () indicates the number of times a given variant or combination of variants was found. S cultures which developed no variants are not given in the table.

TABLE 17.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN Rm CULTURES (24).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (23)	RS (9)	S (4)	Sm (8)	Rp (4)	Sp (2)
	Rm (11)	Rm, R-Rm (3)	Rm (1)	R, Rm (2)	Rm, R (2)	R, S (1)
	Rm, R-Rm (5)	Rm, Rp (1)		Rm, Rm-Sm (1)		
	Rm, Sm (1)	R, R-Rm (1)	Rm, R (1)	Rm (1)	Rm, RS (1)	S, Rp, R, Rm (1)
	R-Rm, RS (1)	Rm, R, Rp (1)	Rm, R, Rp, Sp (1)	Rm-Sm (1)	Rm, Sm, R-Rm, Rm-Sm (1)	
	R-Rm (1)	Rm, R, Sm, Rm-Sm (1)	Rm, Sm, R, Rm-Sm (1)	Rm, R-Rm (1)		
	Rm, Sm, R-Rm (1)	Rm, Sm, S, Rm-Sm (1)		Rm, R, R-Rm (1)		
	Rm, Sm, S, Rm-Sm (1)	R (1)		Rm, R-Rm, Rm-Sm (1)		
	RS, Sm, S (1)					
	Rm, Rm-Sm (1)					

The figure in () indicates the number of times a given variant or combination of variants was found. Rm cultures which developed no variants are not given in the table.

TABLE 18.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN SM CULTURES (26).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (13)	RS (19)	S (22)	Rm (7)	Rp (1)	Sp (1)
	Sm, S (3)	Sm, S (6)	Sm (11)	Rm-Sm, Sm (3)	S, RS, R, Sp, Rp-Sp (1)	S, RS (1)
	S, RS (2)	S (4)	S-Sm, Sm 2)	Rm-Sm, Sm, S (1)		
	Sm, S, RS (3)	S-Sm, Sm, S (1)	S-Sm, S (1)	Rm-Sm, Sm, RS (1)		
	Sm, Rm, S, RS (1)	Sm, S, R (2)	Sm, RS (1)	S-Sm, Sm (1)		
	Sm, Rm (1)	S, R (1)	S, RS (1)	Sm (1)		
	Sm, Rm, S (1)	Sm, Rm, R (1)	S-Sm, Sm, R-Rm (1)			
	Sm, Rm, RS (1)	S, R, Rm-Sm (2)	Sm, Rm, S, RS (1)			
	S, RS, Sp, Rp, Rp-Sp (1)	Sm, Rm-Sm (1)	Sm, RS, Rm-Sm (1)			
		S, R, Sm-Rm (1)	Sm, Rm (1)			
			Sm, R (1)			
			Rm, Rm-Sm (1)			

The figure in () indicates the number of times a given variant or combination of variants was found. Sm cultures which developed no variants are not given in the table.

TABLE 19.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN Rp CULTURES (26).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (4)	RS (6)	S (4)	Rm (0)	Sm (1)	Sp (9)
	Rp (3)	Rp (6)	Rp (2)		Rp (1)	Rp (7)
	R-Rp (1)		Rp, Sp, R (1)			Rp, R (1)
			Rp, S-Sp (1)			Rp-Sp, Rp (1)

The figure in () indicates the number of times a given variant or combination of variants was found. Rp cultures which developed no variants are not given in the table.

TABLE 20.—A SUMMARY OF THE OCCURRENCE OF VARIANTS ARISING IN Sp CULTURES (24).
THE VARIANTS ARE GIVEN AT THE HEAD OF EACH COLUMN AND BELOW IT
THE FREQUENCY OF ACCOMPANYING VARIANTS

Variant Accompanied by	R (2)	RS (4)	S (9)	Rm (1)	Sm (0)	Rp (19)
	Sp (1)	Sp, Rp (2)	Sp (5)	Sp (1)		Sp (7)
	Rp-Sp, Rp, Sp (1)	Sp (1)	S-Sp, Sp (3)			Rp-Sp, Sp (6)
		S-Sp, S, Sp (1)	Rp-Sp (1)			S-Sp, Sp (2)
						Rp-Sp, S (1)
						S-Sp (1)
						Rp-Sp (1)
						Sp, RS (1)

The figure in () indicates the number of times a given variant or combination of variants was found. Sp cultures which developed no variants are not given in the table.

often changed with difficulty to other forms. Hence as the result of the promiscuous formation of variants, the R or more stable form, would be the one form which might ultimately accumulate in the largest numbers.

In this discussion of the dissociation tendencies the considerations of the theory of cycles has been avoided. The application of such a theory to the experimental material here submitted seems impossible. Although six distinct forms (the RS is an intermediate form) have been described and their change from one to the other studied many times, the results point only to reversible changes always involving certain intermediate colony forms which occur regardless of the direction of the reaction. A reexamination of the dissociation chart which apparently represents the facts as regards the dissociation of *B. anthracis*, leaves no apparent possibility for the inclusion of a life cycle.

VIRULENCE

The virulence of the various forms was tested against guinea-pigs and mice as soon as they were isolated and obtained in pure culture. The question of contamination must always be borne in mind when work of this nature is involved. Hence any characteristic property as virulence, is helpful in setting at rest such possible interpretations. For this purpose certain of the earliest virulence tests will be reported here, not so much for a comparison of virulence of the different forms but rather as an indication of the relation of these peculiar colony types to the disease of anthrax.

The R or usual form of *B. anthracis* killed guinea-pigs in about 2 days with the usual gross pathological findings. The R colony form was recovered on plates streaked with the heart blood or a piece of the liver.

The RS and S forms killed when first isolated, in about the same time as the R form with similar gross pathological findings and the respective colony forms were isolated from the dead animals.

The Rm culture killed a guinea-pig in 108 hours with a gross pathology very similar to that usually attributed to anthrax. Plates of both heart blood and spleen gave a pure culture of the Rm form which had lost some of its mucoid property (R-Rm), in the animal passage.

The Sm form injected subcutaneously into a guinea-pig killed the animal after 96 hours with the usual anthrax findings at autopsy. The culture recovered was not the Sm but what appeared to be S and RS colony forms, in equal proportions. The "S" colonies after 48 hours

appeared somewhat mucoid, particularly in the heavy streaks on the plate. Sub-cultures showed that these "S" colonies gave rise to Sm growth on the heavy streaks. Hence in the light of experiments dealing with the important rôle of the reaction of the medium on the colony structure of this form, it is possible that this culture recovered from the animal was the Sm form.

A later re-isolation of the Sm culture from the RS form and immediate inoculation (after purification) into mice showed that it killed the mouse in 44 hours with a recovery of a culture composed of mainly S colony forms with 2 or 3 RS forms and the same number of perfectly typical Sm colony forms. The control on the inoculum plated after the inoculation of the mouse showed only Sm forms to be present.

The Rp form killed an inoculated guinea-pig in 75 hours and a pure culture of the Rp form from the animal was recovered.

The Sp form failed to kill the first guinea-pig with which it was injected but in the next experiment killed 3 mice in an average of 42 hours with the recovery of pure Sp cultures. Subsequent tests have shown that it is able to kill guinea-pigs also.

It was thus early shown that all forms had some degree of pathogenicity and gave the usual gross pathological findings noted in guinea-pigs dead of anthrax. To determine the relative virulence of these various forms many tests, involving both mice and guinea-pigs, have been performed throughout the investigation. It has been found that in every case, except the R form, virulence has diminished with time. It is to be noted that all forms have been carried under exactly the same culture conditions during this period.

Table 21 shows the results of virulence tests in mice performed near the start of this work and at its close. The results I think show clearly the loss in virulence that has occurred in forms associated with the S colony form (S, Sp, Sm and even RS). Also the loss associated with the mucoid property (Rm and Sm). Where these two properties co-exist as in the Sm form the loss occurs most rapidly. It is noted that in the first test the Sm is avirulent for mice, yet 60 days earlier when it was first isolated it had been able to kill guinea-pigs.

This table* brings out the difference in the virulence of the various forms. Virulence has been associated with the smooth colony form in practically all other microorganisms studied from a point of view of dissociation. In the case of anthrax the virulence is apparently asso-

* The differences in virulence of the various forms, as brought out in this table, are representative of the results of similar tests on over 50 guinea-pigs and 150 mice.

ciated with the R colony form. This is in agreement with the major portion of the literature dealing with colony variations and virulence in *B. anthracis*. Preisz⁵ found his homogeneous colony devoid of virulence. Bail and Flaumenhaft¹⁶ and Gratia¹⁹ also found that the smooth colony had less virulence than the rough colony. However, Wagner¹⁷ reports the reverse as a result of a limited number of guinea-pig and mouse inoculations. From the work reported in this paper an explanation for these contradictory results can be offered. In the first place Wagner's "B" colony, from his excellent photographs, does not conform to the ultimate smooth S colony. It is more of the RS type of colony and as such should have a virulence not greatly less than the R form. It is easily conceivable from my own experience, that with

TABLE 21.—CHANGES IN VIRULENCE FOR MICE * DURING PROGRESS OF THE INVESTIGATION

Date	Mouse Number	R Hours	RS Hours	S Hours	Rm Hours	Sm Hours	Rp Hours	Sp Hours
7/ 5/26	1	21	15	18	216	Survived	33	18
	2	24	21	21	264	Survived	69	38
	3	33	33	33	Survived	Survived	Survived	69
	Average	26	23	24	240		51	42
1/25/28	1	25	25	85†	Survived	Survived	25	Survived
	2	25	39	133†	Survived	Survived	65	Survived
	3	25	39	Survived	Survived	Survived	Survived	Survived
	4	39	63	Survived	Survived	Survived	Survived	Survived
	Average	28	41				45	

* Mice were given intraperitoneal injections with 0.25 cc. of a salt solution suspension of an agar culture.

† Plates streaked at necropsy gave no evidence of the presence of *B. anthracis*.

a limited number of animals the variation in the animals might account for the differences reported by Wagner. It is worthy of note that no differences in the pathogenicity is reported for guinea-pigs by Wagner. A second possible error that would account for lack of harmony in virulence experiments involving variant forms of *B. anthracis* is the loss of virulence of the variants after prolonged cultivation with no apparent change in colony form.

VIRULENCE OF THE REVERTED R FORMS

A point of extreme significance is whether or not there is a return of virulence with a return to the colony form associated with virulence. Stryker³⁸ was able to effect a reversion of the pneumococcus to the virulent type from the avirulent form. It is important to note that she found this reversion more difficult, the greater the "fixity" of the variant. Jordan³⁹ has reported a return of virulence in *B. paratyphosus* B.

following a reversion from the R to the S. On the other hand Griffith ³⁹ in the case of pneumococcus has reported a reversion as far as colony form was concerned, but although the recovered colony was practically identical with the initial virulent S form the reverted culture lacked virulence.

In the case of anthrax the results reported here indicate that there may or may not be a return of virulence with a reversion to the R form. The latter appears to be the more prevalent state of affairs as indicated by table 22.

The one definite instance of reversion to a grade of virulence usually met with in the stock R variant is noted in the first case listed in table

TABLE 22.—THE EFFECT OF REVERSION OF THE VARIANTS TO THE R OR RS CULTURE TYPE ON VIRULENCE IN MICE

Initial Form	Reverted Form	Conditions of Reversion	Number of Mice		Average Time in Hours
			Inoculated	Dead	
S	R	6 days in broth 300 cc.	3	3	42
S	Control		4	0	
S	RS	Rabbit lesion*	3	2	70
S	Control		4	2†	
Rm	R	23 days in 10% guinea-pig serum broth	3	2	176
Rm	Control		4	0	
S	R	14 days in broth	4	1	41
S	Control		4	0	
Sm‡	R	7 days in broth under 10% CO ₂	1	0	
S	R	3rd transfer in 10% homol. immune serum	3	0	
S	R	9 days in milk	3	0	
Rp	R	15 days in milk	3	0	

* A rabbit inoculated in the marginal vein of the ear with living S antigen for the purpose of obtaining immune serum, developed a local infection from which the RS form was recovered.

† No evidence in the cadaver or in tissue cultures of anthrax.

‡ In this and the three following cases no controls are given since no return of virulence was noted in the reverted forms.

22. An inspection of virulence tests on the S culture performed at the time that this reversion occurred, disclosed the fact that the S culture apparently had some residual virulence, for it killed irregularly. Hence the apparent return of virulence may be simply the enhancement of a property present in a diminished quantity.

This brings us to the very important consideration of the correlation of virulence and colony properties. The recent work in microbic dissolution has tended to simplify the problem of virulence by suggesting a correlation of virulence and dissociant form as usually determined by culture properties. The work here presented raises the serious question as to the absolute validity of such a correlation. Although this work shows such a correlation in cultures stabilized by continued subculturing, there are a large number of exceptions to be met in variants recently

isolated from virulent forms. Further once a variant has lost its virulence more or less completely, the present work indicates that a reversion to a colony form usually identified with virulence is not proof of a return of the property of virulence.

Such denial of an absolute correlation of virulence and colony form is by no means new. To believe that workers in the past have always failed to observe cultural variation in cultures which have followed the usual course of stock laboratory strains and lost their virulence, is highly improbable. In the case of anthrax vaccine cultures, it is certain that attenuation is often accomplished without marked changes in the colony form of the organism. An examination of the first and second Pasteur vaccines obtained directly from the Pasteur Institute of Paris, revealed colony forms not markedly different from the R form. The first vaccine resembles more closely the R form. It gave a somewhat smaller colony and did not have quite the coarse structure of a typical R. The second vaccine culture gave colonies somewhat similar to the RS form. This observation in no way contradicts the observations of Preisz,¹¹ who found diverse colony forms in the Pasteur vaccines. It is entirely possible, if the current Pasteur vaccine cultures have a common origin with those with which Preisz worked (which is not necessarily to be assumed), that in the intervening time a considerable change with respect to colony form may have been effected in these cultures.

Hence it may be safe to conclude that virulence and colony form may be associated in the case of *B. anthracis*, but that this association is not absolute.

DISSOCIATION IN VIVO

The change from one culture type to another in the animal has been noted many times in the virulence tests that have been performed in this work. Such observations are not new, for Preisz⁵ in 1911 recovered from mice injected with a mucoid variant, not only the mucoid growth but forms which probably agree with the S and R forms. Bail and Flaumenhaft¹⁶ recovered R and S colonies from animals injected with cultures corresponding probably to what is designated as the S form in this paper. More recently Gratia¹⁹ reported a similar experience following the injection of rabbits with a slightly virulent S form.

Table 23 lists twelve examples of in vivo dissociation. In all of these cases, the inoculum was cultured following the inoculation of the animal to make certain of the material actually injected into the animal. After the death of the animal a bit of the liver was streaked on an agar

plate which was examined and the results recorded after 24 hours incubation.

A point of interest is that in the dissociation within the animal body the trend of the reaction conforms to the scheme (dissociation chart) suggested to cover dissociation *in vitro*.

In all of the above cases dissociation *in vivo* was recognized only after the death of the animal. In order to make certain that these changes could take place while the animal was still alive the following experiment was performed.

Exper. V 26.—A guinea-pig was inoculated under the skin with 1 cc. of a salt solution suspension of an Rm culture. This Rm culture had been recently isolated (6 generations) from the R form and was still virulent. At stated intervals a small sample of heart blood was drawn and plated. As soon as a subcutaneous swelling at

TABLE 23.—EVIDENCE OF *IN VIVO* DISSOCIATION

Test	Animal	Inoculum	Dead in Hours	Variant Recovered
V-7	Mouse	Rm	216	R 100
V-7	Mouse	Rm	264	R 100
V-8	Mouse	Sm	71	S-Sm 100
V-10	Guinea-pig	Rm	90	Rm 10%, R-Rm 90%
V-12	Guinea-pig	Sp	239	S 80%, Sp 20%
V-14	Guinea-pig	Sp	77	S 100
V-16	Guinea-pig	Sp	189	Sp 20%, Sp-RS 70%, S 10%
V-16	Guinea-pig	Sp	127	Sp 98%, S 2%
V-16	Guinea-pig	Sp	125	Sp 99%, R 1%
V-17	Mouse	Rm	35	Rm 5%, R 95%
V-25	Mouse	Sm	44	Sm 0.5%, RS 0.5%, S 99%
V-43	Mouse	Rp	25	Rp 99%, RS 1%

the point of inoculation developed a small amount of the fluid from this wound was obtained with the aid of a large bore needle and plated. The results are given in table 24.

SUMMARY

As a result of aging a laboratory stock culture of *B. anthracis* under various culture conditions, six additional culture types have been obtained. These forms have all been reisolated from the usual anthrax colony type, "purified" by over 100 serial single colony isolations. After a similar "purification" all variants have been caused to revert to the usual colony type.

Colony characteristics of all forms have been described and terms of reference suggested. The form giving rise to the usual rough, grey colony has been designated as the R form. One variant formed small, smooth, raised colonies and has been termed the S form. The form intermediate between these two, with respect to its colony formation, has been called the RS form. Two colony types similar to the R and S

forms with respect to roughness or smoothness, but having an additional mucoid property have been designated as the Rm and Sm forms respectively. Similarly two forms which gave very thin growths on agar plates have been designated as the Rp and Sp forms respectively.

Morphologically the S and Sm forms are shorter and often slightly wider than the R or Rm forms. The former frequently show vibrio forms as contrasted to the latter. In agar cultures 24 to 48 hours old the S forms frequently show coccoid forms. The phantom cultures show characteristic swellings or involution forms which increase in numbers as the cultures become older. All forms were nonmotile. Spores were observed in all forms. In the course of continual transfers, however, the mucoid and phantom forms have become nearly asporogenic. Spores are readily observed in the R, RS and S forms.

TABLE 24.—IN VIVO DISSOCIATION OF THE Rm FORM IN A GUINEA-PIG

Time in Hours	Heart Blood	Site of Inoculation
24.....	Sterile	
48.....	Sterile	
68.....	1 R colony	Contamination, no anthrax
92.....	1 Rm colony	1 colony (contamination)
116.....	6 Rm colonies	40 Rm colonies, 2 R colonies
121.....	Dead *	Rm 70%
	Rm 99%	RS 10%
	R 1%	Rm-Sm 10%
		R 10%

* The necropsy was performed within 5 minutes after death.

Only slight quantitative differences existed between the forms when grown on various differential mediums as gelatin, milk, milk agar, blood agar, and Russell's double sugar medium. No indol or H_2S was produced by any form. Manometric curves (cultures in 100 cc. h-tubes) were practically identical in the case of the R, RS, S, Rm and Sm forms. The curves for the phantoms had a diminished slope. Respiratory quotients for all forms on plain agar were similar.

A striking effect of medium upon the development of the mucoid property of the Rm and Sm forms was demonstrated. Agar with a P_H of 7.0 was unfavorable for the development of this property. Agar with a P_H of 7.8 was favorable. Nutrient agar with a P_H of 7.0 was favorable if the growth took place under 10 to 15% CO_2 . It was suggested that the dissolved or combined CO_2 is the factor influencing the production of this striking mucoid property.

Glucose added in as small amounts as 0.10% seriously inhibits the production of the mucoid property even on medium with an initial P_H of 7.8.

Dissociation of the variants has been studied under various conditions; temperature of 5, 12, 26, 37 and 42.5 C; liquid and solid mediums; broth in 10 cc. and 300 cc. volumes; broth of various reactions— P_H of 6.3, 7.4 and 8.2; broth to which had been added 2% glucose, 0.1% phenol, 10% normal rabbit serum, 10% normal guinea-pig serum, 10% normal rat serum; broth prepared without the addition of peptone or salt; milk; and filtrates of old cultures. In general it may be said that dissociation occurs under conditions where actual multiplication of the organism take place; and further that these conditions are of a nature that would be considered unfavorable for the optimum development of the organism.

Certain definite trends of dissociation of *B. anthracis* have been indicated schematically and evidence in favor of this scheme submitted. All changes in colony form appear to be reversible and gradual. No evidence of a cycle has been noted.

As a result of numerous virulence tests on mice and guinea-pigs, it has been observed that all variants were able to produce death in these animals when first isolated from the R form. After many transplants ($1\frac{1}{2}$ years) on agar the S, Rm, Sm and Sp forms are practically avirulent, the RS form has decreased slightly in virulence while the R form remains unchanged with respect to its killing power.

R forms derived from avirulent forms are not virulent. R forms derived from slightly virulent forms had an increased virulence approximating that of the original R form.

Dissociation *in vivo* has been demonstrated particularly in the case of the Rm, Sm, Rp and Sp forms.

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Science in the University of Michigan.

